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NOTE ON THE PREPARATION OF P-DIMETHYLAMINO-BENZALDEHYDE.

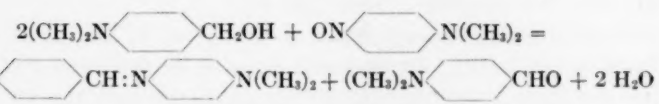
By T. INGVALDSEN and L. BAUMAN.

(*From the Department of Internal Medicine, State University of Iowa, Iowa City.*)

(Received for publication, December 12, 1919.)

During the war there was a shortage of *p*-dimethylaminobenzaldehyde. This compound is the chief ingredient of Ehrlich's reagent which is used for the determination of urobilinogen in the urine, feces, and bile. As there appeared to be a demand for this substance by physicians and investigators it was desirable to attempt to simplify the method of preparation. Several methods are to be found in the literature. The method of Ullmann and Frey[1] appeared simple and easy of execution. It consists of the following steps: (*a*) The preparation of *p*-nitrosodimethylaniline; (*b*) the reaction of (*a*) with *p*-dimethylaminobenzyl alcohol; (*c*) the cleavage of anhydro-*p*-dimethylaminobenzaldehyde-*p*-aminodimethylaniline and liberation of the desired aldehyde.

The reaction involved in step (*b*) is as follows.



As an excess of the nitroso body is used, the second molecule of aldehyde is partially converted into the benzylidene body.

Experience has led us to modify the procedure in several respects. The isolation of the hydrochloride of the benzylidene compound is omitted. The decomposition of the free base by formaldehyde and acetic acid is carried out at room temperature. The crude aldehyde is purified by distillation in a partial vacuum.

[1] Ullmann, F., and Frey, B., *Ber. chem. Ges.*, 1904; xxxvii, 855.

Preparation of p-Dimethylaminobenzaldehyde.

300 gm. of technical dimethylaniline are dissolved in 1,500 cc. of dilute hydrochloric acid (1:1) and placed in a freezing mixture. A saturated solution of 180 gm. of sodium nitrite in water is added slowly from a dropping funnel while the reaction mixture is being agitated by a mechanical stirrer. The operation requires about 1 hour. The nitroso body is filtered with suction and washed with the dilute hydrochloric acid. In the meantime 360 gm. of dimethylaniline, 250 cc. of formaldehyde, and 600 cc. of concentrated hydrochloric acid are mixed in a large beaker which is heated on the boiling water bath for about 10 minutes after which the nitroso compound is added at once. The violent reaction which ensues is completed in about 5 minutes. After cooling and diluting with water, the base is precipitated by the addition of commercial sodium hydroxide (lye) until all the red color has disappeared. The solid is removed by filtration and washed with tap water.

The moist base is transferred to a heavy beaker and covered with 2,000 cc. of 50 per cent acetic acid and 500 cc. of commercial formaldehyde. The mixture is stirred until 20 minutes after the benzylidene compound has gone into solution. The aldehyde separates as a crystalline mass after adding 1,000 cc. of water and crushed ice. After 12 hours refrigeration the solid is filtered off and washed until the washings are perfectly clear. It is dried at room temperature. Yield 260 gm.

Purification is carried out by distillation of the *dry* aldehyde from an oil bath. At 43 mm. pressure it distills at 200°. The hot distillate is treated with 200 cc. of 95 per cent alcohol, poured into a large mortar, and stirred while distilled water is gradually added. All large lumps must be broken up with the pestle. The product is then transferred to a large beaker containing 1,000 cc. of water and 25 cc. of glacial acetic acid. Yield 250 gm. of pure product melting at 73°C. The approximate cost is about 5 cents per gm.

NOTE ON THE OXIDATION OF SUGARS BY MERCURIC ACETATE IN THE PRESENCE OF AMMONIA.

By T. INGVALDSEN and L. BAUMAN.

(From the Department of Internal Medicine, State University of Iowa, Iowa City.)

(Received for publication, December 12, 1919.)

In the past a number of investigators have studied the oxidation of sugars by mercuric salts with the object of obtaining a method for their qualitative or quantitative determination. Herzfeld[1] found that glucose is readily oxidized by red mercuric oxide in the presence of barium hydroxide. Heffter[2] obtained gluconic acid by boiling a glucose solution with yellow mercuric oxide.

Ammonium gluconate and ammonium galactonate may be obtained in a 50 per cent yield by the action of mercuric acetate on glucose or galactose in the presence of ammonia. Mannose and lactose are also oxidized by this method but the ammonium salts of their respective acids cannot be isolated in crystalline form.

Preparation of Ammonium Gluconate and Galactonate.

10 gm. of glucose dissolved in 100 cc. of water are treated with 25 gm. of mercuric acetate and 15 cc. of concentrated ammonia solution and allowed to remain at room temperature over night. The solution is then placed on the water bath for 12 hours, saturated with hydrogen sulfide, filtered, purified with bone-black, and evaporated in a partial vacuum. Ammonium gluconate crystallizes when the remaining syrup is treated with alcohol. The crystals occur as thin hexagonal plates and melt at 155–157° (uncorrected). The physical constants are similar to those obtained by Irvine, Thomson, and Garrett[3] and also to those of a

[1] Herzfeld, A., *Ann. Chem.*, 1888, ccxlv, 27.

[2] Heffter, A., *Ber. chem. Ges.*, 1889, xxii, 1049.

[3] Irvine, J. C., Thomson, R. F., and Garrett, C. S., *J. Chem. Soc.*, 1913, ciii, 238.

sample of ammonium gluconate obtained by the oxidation of glucose with bromine. The yield is 6 gm. from 10 gm. of glucose. The recrystallized substance analyzed as follows.

0.4156 gm. required 19.5 cc. of 0.1 N sulfuric acid (Kjeldahl).
Calculated for $C_6H_{15}O_7N$ 6.57 per cent. Found 6.56 per cent.
The specific rotation was + 13.63°.

$$[\alpha]_D^{20} = \frac{6.3192 \times +3.21°}{1.3642 \times 1.0908}$$

Ammonium galactonate when prepared by the above method crystallizes in small needles. The yield is 7 gm. from 10 gm. of galactose. The melting point is 155–157°.

The recrystallized sample analyzed as follows.

0.3388 gm. required 15.9 cc. of 0.1 sulfuric acid (Kjeldahl).
Calculated for $C_6H_{15}O_7N$ 6.56 per cent of nitrogen. Found 6.57 per cent.
The specific rotation was + 3.33°.

$$[\alpha]_D^{20} = \frac{6.3116 \times +0.75°}{1.2986 \times 1.0946}$$

FAT-SOLUBLE VITAMINE.*

IV. THE FAT-SOLUBLE VITAMINE CONTENT OF GREEN PLANT TISSUES TOGETHER WITH SOME OBSERVATIONS ON THEIR WATER-SOLUBLE VITAMINE CONTENT.

By H. STEENBOCK and E. G. GROSS.

With the Cooperation of Mariana T. Sell.

(*From the Laboratory of Agricultural Chemistry, University of Wisconsin, Madison.*)

(Received for publication, December 6, 1919.)

While the mere isolation and the determination of properties of substances found in the plant and animal kingdom are most fascinating and stimulating procedures in the chemical laboratory, such work becomes especially engaging from the biochemical point of view, when speculation as to the rôle of the multitudinous array of compounds is injected into the work. Viewed from this angle, there is little wonder that the attention of many biochemists should, at devious times, have been absorbed in the work of isolating organic acids, carbohydrates, proteins, alcohols, esters, and bases with the prospect of securing an idea as to the origin, the interrelations, and the fate of these substances.

In animal physiology, work of this nature has yielded far reaching results in the formulation of our present day conceptions of the relations of the individual to his nutritional environment. Especially is this evident in problems pertaining to energy relations and those concerned with the constructive process of growth and maintenance. But, as these have been developed, it has become increasingly evident that, if our conception of physiological processes in various fields is to be built up symmetrically, it becomes imperative that information on the nature of the vitamines be accumulated so that their specific rôle in the animal body can be determined. Funk (1), and Braddon and Cooper (2), who sur-

* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

mised a relation between polyneuritis as caused by an insufficiency of the water-soluble vitamine and carbohydrate metabolism, and then Drummond (3), who investigated the effect of the water-soluble vitamine on nitrogen metabolism and the effect of the fat-soluble vitamine on fat metabolism, are the only investigators—as far as we know—who sought to associate the rôle of vitamines with the general body metabolism.

It is true that much of immediate practical importance in vitamine relations may be gained by a study of the dietary properties of various foodstuffs fed singly and in combinations, but it is not to be questioned that ultimately a true conception of problems in nutrition is dependent on the determination of the occurrence of various substances in foods and a development of an appreciation of their physiological rôle. We refer here to the effect of various substances on secretion, motor activity, irritability, conductivity, permeability, and cell proliferation all of which are concerned in such a gross physiological process as growth.

In the field of vitamine physiology, progress in the suggested direction is beset with many difficulties as shown by the fact that no vitamine has as yet been isolated. If it is not the lability of the compound in question, it is its extreme chemical indifference to such reagents as are ordinarily used to modify solubilities that prevents its separation from its environment. The present state of our knowledge is such that any indications as to the probable nature of a vitamine is worthy of investigation. In the case of the fat-soluble vitamine we have adopted as our working hypothesis (4) that it is either identical with or else chemically related to certain yellow plant pigments. With this as a clue, which is an outgrowth of their often observed association in nature (5), methods leading to the extraction of certain yellow pigments have given us many pigmented solutions carrying the fat-soluble vitamine. While this appears promising, it seemed imperative—before developing this work extensively—to accumulate information as to the best sources available for the isolation of this dietary essential and also to determine its stability in such materials.

Unfortunately data on the distribution of the fat-soluble vitamine are very limited in the literature, and, such as there are, were to a large extent obtained when the laboratory technique of determining dietary relations was not so highly developed as it is

at the present time. This situation has been discussed by Osborne and Mendel (6) in a preliminary communication in which they have submitted some data, but here again their data are not directly comparable with ours so that we have been obliged to continue with our accumulation of information to bring out what we shall present later. In the present paper we present data on the fat-soluble vitamine content of alfalfa, clover, spinach, lettuce, cabbage, and chard, and in addition present some data on their water-soluble vitamine content as well.

EXPERIMENTAL.

As in the previous papers of this series (5, 7, 8) the relative amount of fat-soluble vitamine present in the various plant tissues was determined by establishing the minimum amount—down to 5 per cent of the ration—necessary to satisfy the growth impulse of a recently weaned young rat for a period of at least 4 months. Using this technique it is, of course, necessary to have the young animals, as near as possible, of the same age and in good condition at the start of the experiment. Furthermore, it is imperative to know that in the experimental ration all other dietary requirements are complied with, so that growth is possible when a deficiency in the fat-soluble vitamine content does not occur or when it is corrected. Such a ration is readily formulated in a basal mixture consisting of purified casein, dextrin, agar, salts,[1] and water-soluble vitamine. The latter was usually incorporated as an alcoholic solution of ether-extracted wheat embryo, but sometimes as ether-extracted wheat embryo itself. As a rule, four experimental animals were fed in a group to eliminate individual differences by the law of averages.

Fat-Soluble Vitamine Content of Alfalfa.

Probably more of the statements relative to the fat-soluble vitamine content of leafy materials are based on data obtained with alfalfa than with any other materials, but unfortunately one is unable to glean from the information available, as pointed out

[1] For composition of salt mixtures see Steenbock, H., Boutwell, P. W., and Kent, H. E., *J. Biol. Chem.*, 1919, xxxv, 517; Steenbock, H., and Gross, E. G., *J. Biol. Chem.*, 1919, xl, 501.

by Osborne and Mendel (6), whether failure of growth, when it occurred, was due to lack of the fat-soluble or the water-soluble vitamine. These complications are avoided in the data presented in our Charts 1 to 5. The alfalfa used in these experiments was an early summer growth cut in full bloom when about 18 inches high. It was cured at room temperature in the diffuse light of the laboratory and was then ground to a meal.

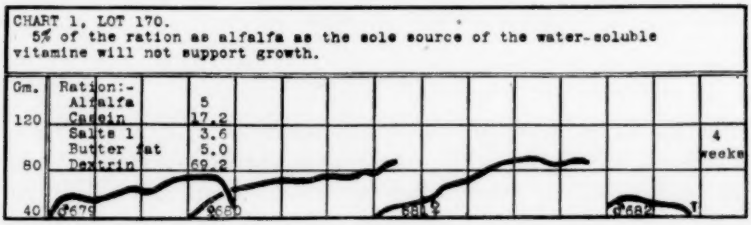


CHART 1.

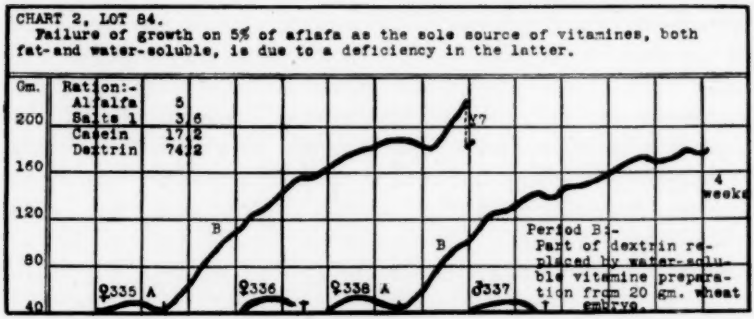


CHART 2.

Charts 1 and 2 demonstrate the deficiency of alfalfa in the water-soluble vitamine; in fact Rats 679 and 682 both went into convulsions as the result of polyneuritis. That the inhibiting factor really was the water-soluble vitamine is shown by the response in growth of Rats 335 and 338 when this dietary essential was added in an alcoholic extract of wheat germ.

In Chart 3 it is seen that growth is possible at the normal rate on a ration carrying 5 per cent of alfalfa as the source of vitamines if the water-soluble vitamine deficiency is corrected. Fur-

thermore, young were successfully reared. Rat 683 raised two young out of a litter of seven to an average weight of 42 gm. in 32 days. While the time for rearing was prolonged 8 to 10 days beyond the normal, the fact that the rearing of young was possible gives indisputable evidence of the richness of alfalfa in the fat-soluble vitamine as the other ingredients of the ration have repeatedly been shown to be free from it. How much less than 5 per cent of the ration might have been constituted of alfalfa and

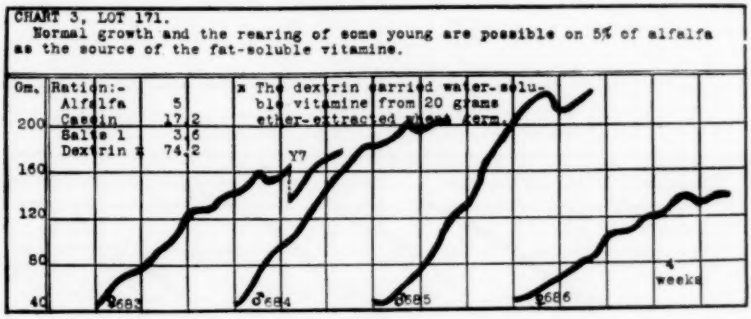


CHART 3.

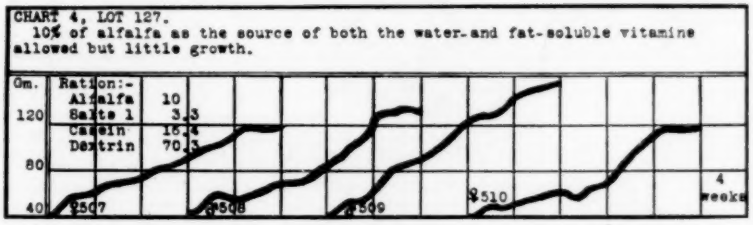


CHART 4.

still have produced results such as the above we have not determined, but we are inclined to think that with our material we had reached approximately the lowest level possible. This is suggested by the curves of growth and the behavior of the young.

Having established that normal growth and the rearing of some young are possible on 5 per cent of alfalfa no question of a fat-soluble vitamine deficiency could be raised in the case of a ration containing 10 per cent of alfalfa, as shown in Chart 4. Failure to grow at the normal rate must be attributed to a lack of a sufficient

amount of the water-soluble vitamine as in the lots pictured in Charts 1 and 2. No symptoms such as the convulsions of polyneuritis were observed nor were they expected as animals growing at the rate indicated will often continue their subnormal rate of growth for many months with no signs of collapse.

When the amount of alfalfa was increased to 15 per cent, Chart 5, the requirements of the rat for both the fat- and water-soluble vitamines were satisfied; normal growth and the rearing of young became possible. Rat 342 raised three young to an average weight of 43 gm. in 4 weeks.

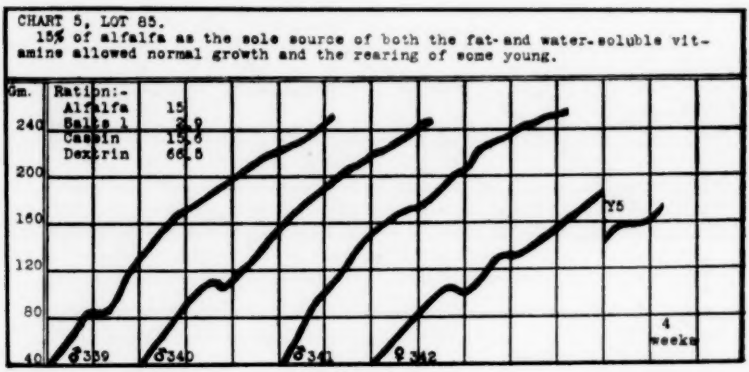


CHART 5

Fat-Soluble Vitamine Content of Clover.

The clover used in these experiments was red clover which had completed its growth and almost its blossoming as only an occasional red blossom appeared among the numerous turning heads. It was dried in the laboratory at room temperature, then ground to a meal, and as such incorporated in the ration.

Chart 6 shows definitely that our clover fed at a 5 per cent level like the alfalfa (Chart 1) did not introduce sufficient water-soluble vitamine into our basal ration to allow growth. The fat-soluble vitamine on the other hand was introduced in considerable amounts with this amount of clover as is evident from the prompt inception of growth in Rats 2328, 2329, and 2331 when the water-soluble vitamine deficiency was corrected by the addition of an

alcoholic extract from ether-extracted wheat embryo. Rat 2330 had died as a result of lack of the water-soluble vitamine before the addition was made.

In Chart 7 are shown the curves of growth of rats on 5 per cent of clover as the source of fat-soluble vitamine in the ration when the water-soluble vitamine deficiency had been corrected from the

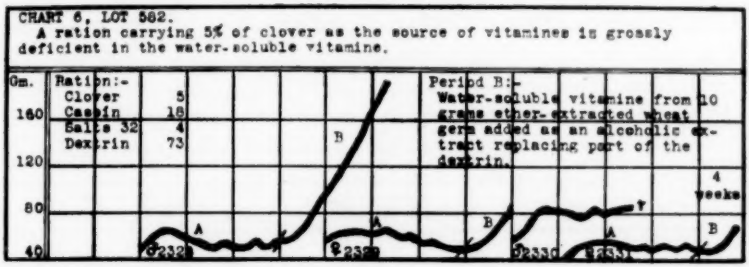


CHART 6.

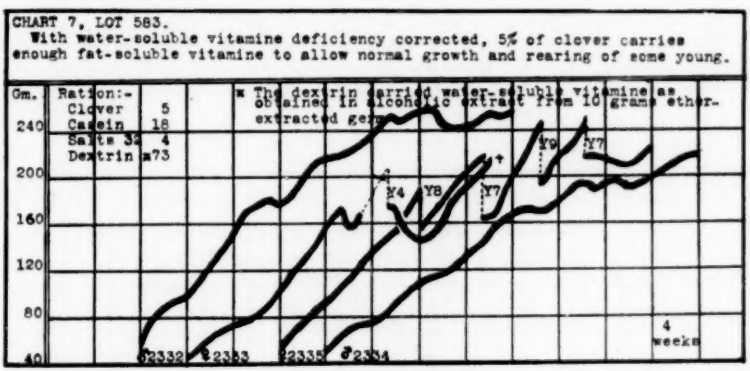


CHART 7.

very start of the experiment by an extract of wheat germ. Growth approximating normal was the result and, as in the case of alfalfa when fed at this level, young were reared though at a subnormal rate of growth. By way of illustration, Rat 2333 raised two out of a litter of four weighing 6 gm. as an average to an average weight of 36 gm. in 9 weeks. They were very small for their age as they should have had this weight when 3 weeks old, but they continued to grow on the ration and when discarded were rated as being

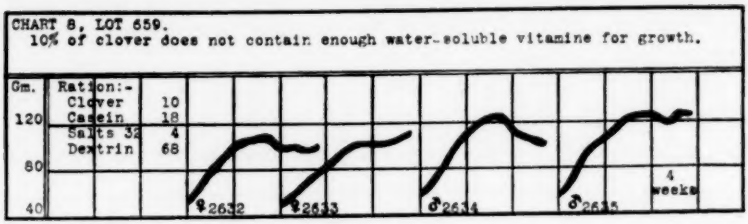


CHART 8.

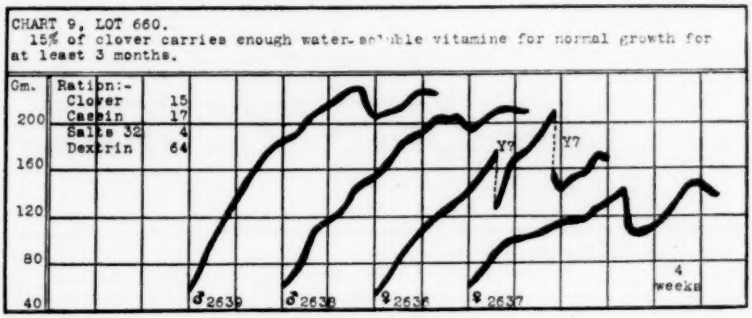


CHART 9.

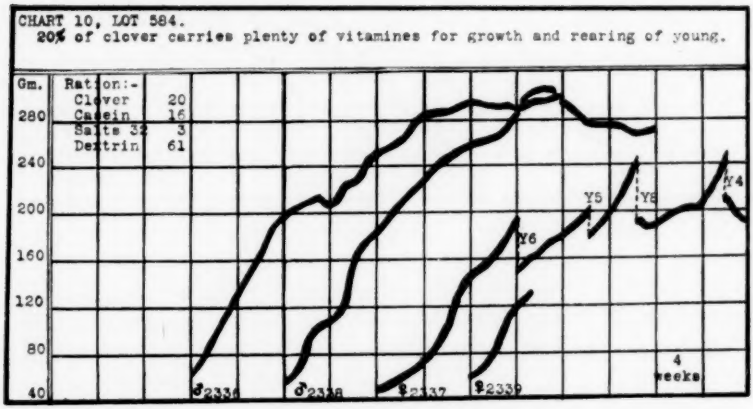


CHART 10.

in good condition. Rat 2335 did not raise her first three litters, but two out of her fourth litter were raised in 6 weeks and 4 days to an average weight of 42 gm., which again is distinctly subnormal.

Having established the dietary efficiency of clover as a source of the fat-soluble vitamine when fed at a 5 per cent level, especially for growth, it must be concluded that the failure of growth when the clover was fed at a 10 per cent level as supplemented in our standard basal ration (Chart 8) must have been due to a water-soluble vitamine deficiency. When increased to 15 per cent (Chart 9) the rate of growth approached the normal. It was not, however, until 20 per cent of the ration was constituted of dried clover that growth was entirely satisfactory and that young were successfully reared (Chart 10). Rat 2339 was accidentally killed by the attendant, but Rat 2337 raised four of her litter of eight to an average weight of 44 gm. in 4 weeks. Her first and second litters were not raised which may, however, have been due to negligence as after parturition she was not segregated from the other members of this group. Under such conditions a perfectly healthy, well nourished animal sometimes discontinues nursing her young and death results from starvation.

Fat-Soluble Vitamine Content of Cabbage.

For these experiments cabbage as purchased on the local market was cut up fine and dried in the laboratory in an air current at room temperature. When in air-dried condition it was finally desiccated over $CaCl_2$ and then ground to a flour for incorporation in the ration.

The growth of rats on 5 per cent of this cabbage as the source of water-soluble vitamine shown in Chart 11 bears testimony that cabbage is not very rich in this vitamine; yet as the curves of growth are better than those where 5 per cent of alfalfa and clover (Charts 1 and 6) was the source of this vitamine, it is possible that cabbage may contain more of it. In spite of the comparatively greater initial weekly increments of growth, when failure threatened to ensue, decline in weight was prompt and decisive and moreover in two of the animals, Rats 715 and 716, observed convulsions of polyneuritis left no doubt as to the exact status

of their nutritive condition. With cabbage increased in amount to 15 per cent of the ration (Chart 12) the degree of growth and its maintenance were much improved and in three of the four animals it could be considered normal. Young were not reared.

As a source of the fat-soluble vitamine we had little success in demonstrating that cabbage is very efficient for probably a number of reasons. For one thing, cabbage when fed at high levels

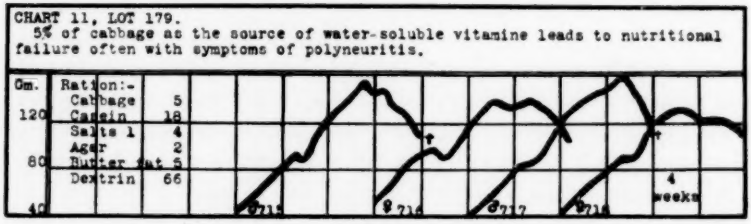


CHART 11.

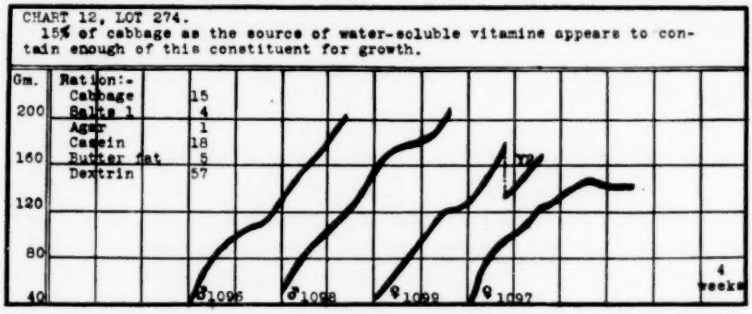


CHART 12.

is very liable to cause digestive disturbances which result in a decreased food intake with therefore a complication in the number of factors operative in the inhibition of growth. With 15 per cent of the ration constituted of cabbage, Rats 668, 669, and 670 were able to grow at a subnormal rate and maintain themselves without any of the secondary symptoms of a fat-soluble vitamine deficiency for 26 weeks which argues strongly for the presence of considerable amounts of this vitamine, yet when the amount was increased at this point by the addition of butter fat a prompt ac-

celeration in the rate of growth occurred (Chart 13). From our data we are inclined to conclude that cabbage is not a good material for the isolation of the fat-soluble vitamine, especially as with its extraction unpalatable volatile oils are removed in large amounts.

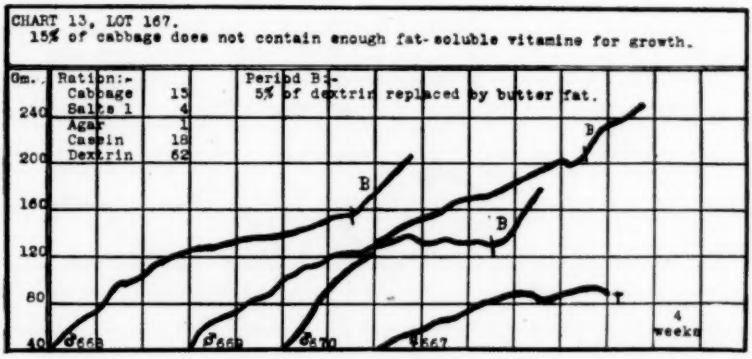


CHART 13.

Fat-Soluble Vitamine Content of Lettuce, Spinach, and Chard.

The plant materials lettuce, spinach, and chard as used in these experiments represented the stems as well as leaves of the plants as sold on the markets for household use. They were dried at room temperature in an air current and ground to a meal.

As the amounts of the above materials were limited, our observations here are not so extensive as with alfalfa and clover. Our technique was also modified in that water-soluble vitamine in all the rations was incorporated as ether-extracted wheat embryo—since from previous experience we had reason to believe that these materials were deficient in it. From the fat-soluble vitamine standpoint the use of wheat germ was entirely justifiable as with it none of this vitamine appeared to be introduced in the ration (Chart 14).

Charts 15, 16, and 17 show that 5 per cent respectively of lettuce, spinach, and chard in our basal ration furnishes enough fat-soluble vitamine for long continued though somewhat subnormal growth. Few young were produced and none was reared, but in no case

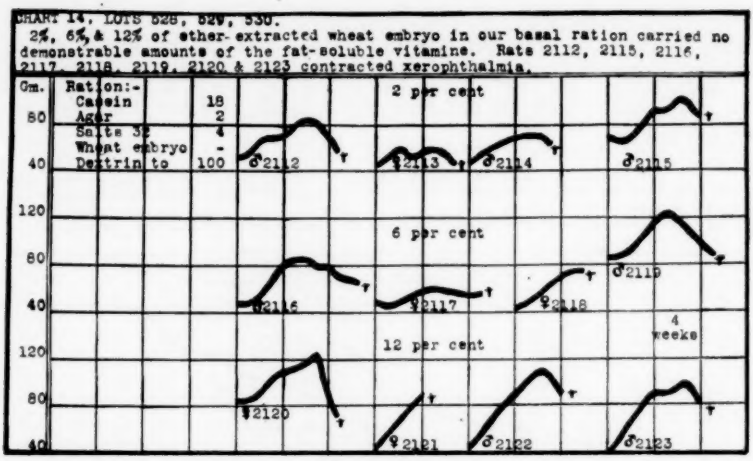


CHART 14.

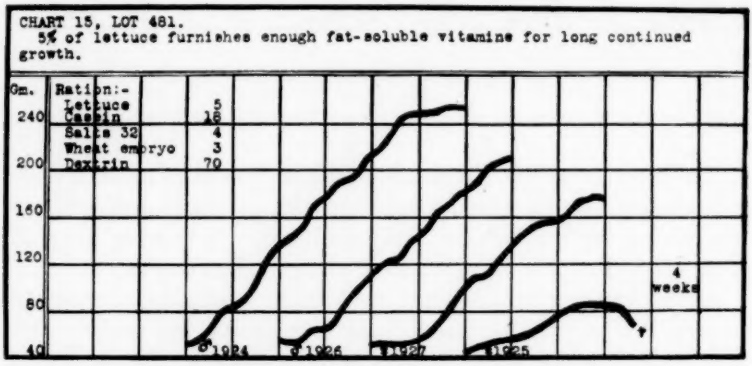


CHART 15.

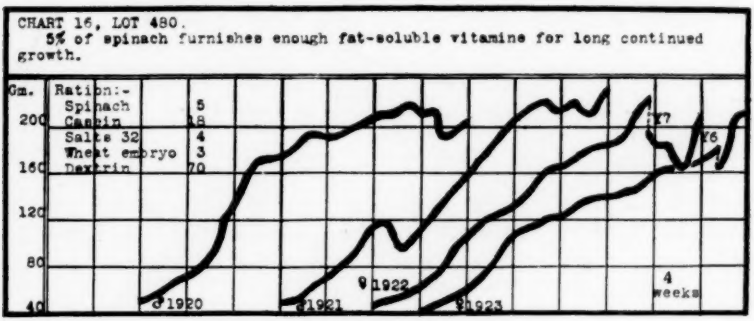


CHART 16.

was there any evidence of xerophthalmia. As judged by appearance, which is far more indicative of an animal's condition than evidence of weight relations alone, the spinach group was in the best condition and the lettuce the poorest.

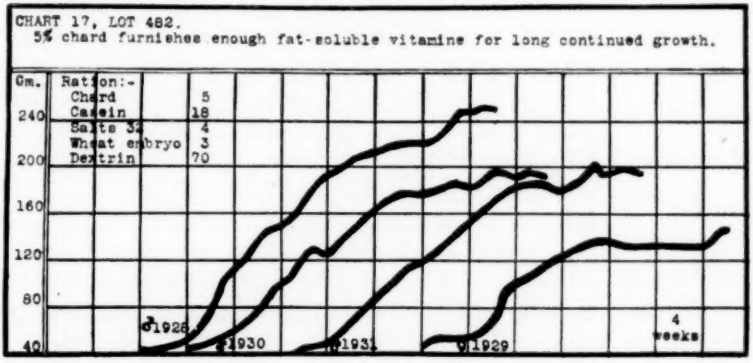


CHART 17.

DISCUSSION.

Whatever may be the reason in the physiological economy of the plant, it appears that of the plant structures the leaves are generally richest in the fat-soluble vitamine, some roots are next in order and last, at least of those investigated, are grains. In this we can see no correlation in the occurrence of the vitamine and the storage function as repeatedly enunciated by McCollum (9), but on the other hand we are assuming for our working hypothesis that where certain yellow plant pigments occur there we may look for the presence of the fat-soluble vitamine. In harmony with this, it is seen that cabbage in the head, containing little pigment, is not to be compared in physiological activity with the other leafy substances and of these latter, lettuce, also somewhat etiolated, is the poorest. Further details of this will be presented later as work now in progress is completed.

SUMMARY.

5 per cent of clover or alfalfa as the sole source of fat-soluble vitamine in a ration, when other dietary requirements are satisfied, allows normal growth and the rearing of some young.

Lettuce, spinach, and chard contain fat-soluble vitamine in amounts of similar magnitude, but of these lettuce may be the poorest.

In harmony with our theory of fat-soluble vitamine distribution and the occurrence of certain yellow plant pigments cabbage does not contain much of this vitamine as is shown by the poor results obtained even when fed at a 15 per cent level.

While 10 per cent of alfalfa and clover was inefficient, 15 per cent of either furnished enough water-soluble vitamine for normal growth; 20 per cent of clover gave still better results.

Cabbage furnished enough water-soluble vitamine when fed at a 15 per cent level.

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FAT-SOLUBLE VITAMINE.*

V. THERMOSTABILITY OF THE FAT-SOLUBLE VITAMINE IN PLANT MATERIALS.

By H. STEENBOCK and P. W. BOUTWELL.

With the Cooperation of Mariana T. Sell and E. G. Gross.

(From the Laboratory of Agricultural Chemistry, University of Wisconsin, Madison.)

(Received for publication, December 23, 1919.)

In the immediately preceding paper of this series (1) we have stated the necessity of securing definite knowledge of the stability of the fat-soluble vitamine as one of the preliminaries leading to its isolation. In regard to the stability of this vitamine there appears to be no unanimity of opinion. Osborne and Mendel (2), and McCollum and Davis (3) are sponsors for the statement that it is thermostable—though the former observed one instance of its destruction by aging—while Steenbock, Boutwell, and Kent (4), and Drummond (5) have shown that it is not so stable as is generally accepted, in fact the latter was inclined to believe that he was dealing with an enzyme-like compound. While we are not inclined to subscribe to Drummond's surmisal, yet we have obtained abundant evidence that under certain conditions this vitamine is very labile, but we also have available evidence that under other conditions it may be very stable. These differences can be readily reconciled as it would be surprising indeed if variations in the stability of this substance should not occur in the very varied chemical environment in which it has been found.

The first information in this connection was presented by McCollum and Davis (3) who found extracts of egg still efficient though the yolks had previously been coagulated by heat. Osborne and Mendel (2) suspected that the impotency of commercial lard might be due to the rather drastic heat treatment to

* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

which the pig tissues had been subjected in the process of extraction, but this was found unwarranted as lard prepared by them in the laboratory with minimum exposure to heat was found just as inactive. Furthermore, butter fat treated with live steam for $2\frac{1}{2}$ hours was still found a source of the vitamine, though it is not stated how much was required to demonstrate that it was present in adequate amounts. Subsequently these same investigators (6) report that butter fat kept at temperatures of 8–18° in the dark and also when exposed to the light did not lose its vitamine. Butter oil obtained by removal of the harder fats from butter by crystallization from warm absolute alcohol, on the other hand, gradually lost its activity even when kept at 8° and in the dark. Drummond (7) found that cod liver oil and whale oil, both of which had been obtained by steam digestion of the tissues, were still very active and therefore surmised that the vitamine was thermostable. Later, however, he substantiated the findings of Steenbock, Boutwell, and Kent (4) that its thermostability in fats such as butter is not very great. Osborne and Mendel (8) have shown that pig tissues such as liver, heart, and kidney contained the fat-soluble vitamine even though they had been dried at 90° for some hours.

In this brief résumé of the literature it is seen how little the matter of the stability of the fat-soluble vitamine has been investigated. It is believed that the data of the different investigators in this field are substantially true to fact and that the variance of opinion is due to a failure to appreciate that the reaction of destruction may be one of low velocity impeded or accelerated by secondary factors. In a ration containing twice as much vitamine as necessary for normal growth no evidence of its destruction would of course be obtained even though one-half of the amount present might have been destroyed by the treatment. In addition, there is no doubt that there obtains a great difference in the stability of the vitamine as found in different materials. This is brought out in the comparison of our previous data with those in the present paper in which are presented data on its thermostability in various plant materials.

EXPERIMENTAL.

The experimental technique used in these experiments was substantially the same as outlined in other publications of this series (1, 4, 9, 10) to which the reader is referred for details. Rats were used as the experimental animals. Shortly after weaning, they were placed on a diet which in its mixture of ingredients was known to satisfy all the dietary requirements of the animal except for the fat-soluble vitamine. The introduction of this latter component was left to that moiety of the ration which consisted of the heated plant materials. These were prepared by autoclaving them for 3 hours at approximately 15 pounds pressure—after soaking in water as they were all air-dried—and then drying them at room temperature in an air current. It was expected that by heating in the autoclave considerable amounts of the vitamine had been destroyed and with this in mind it was thought preferable to dry the materials finally at room temperature rather than in an oven. The growth curves are presented as evidence of the efficiency of the ration in furnishing the requisite amount of the fat-soluble vitamine.

In Chart 1 are presented data obtained with yellow maize, chard, and alfalfa. The maize was of the variety known as Golden Glow or Wisconsin No. 12 and was part of a lot of which the vitamine content had been determined (10). At that time reasons were given for concluding that yellow maize contains just sufficient fat-soluble vitamine to allow normal growth in some animals, but not in all. With this in mind the observed rate of growth of Rats 2503 and 2500, which is of normal proportions, has considerable significance. The somewhat subnormal rate of Rats 2501 and 2502 does not detract from this, as, by the law of averages, it is what might have been expected with animals fed on the unheated maize due to variations in the ability to grow on slightly deficient diets. It is believed safe to conclude that none of the fat-soluble vitamine of yellow maize was destroyed by autoclaving at 15 pounds pressure for 3 hours.

Chard as one of the leafy plant materials has been shown to be comparatively rich in its content of the fat-soluble vitamine (1); 5 per cent being sufficient in a low calorie ration for long continued growth. Autoclaving does not appear to result in a

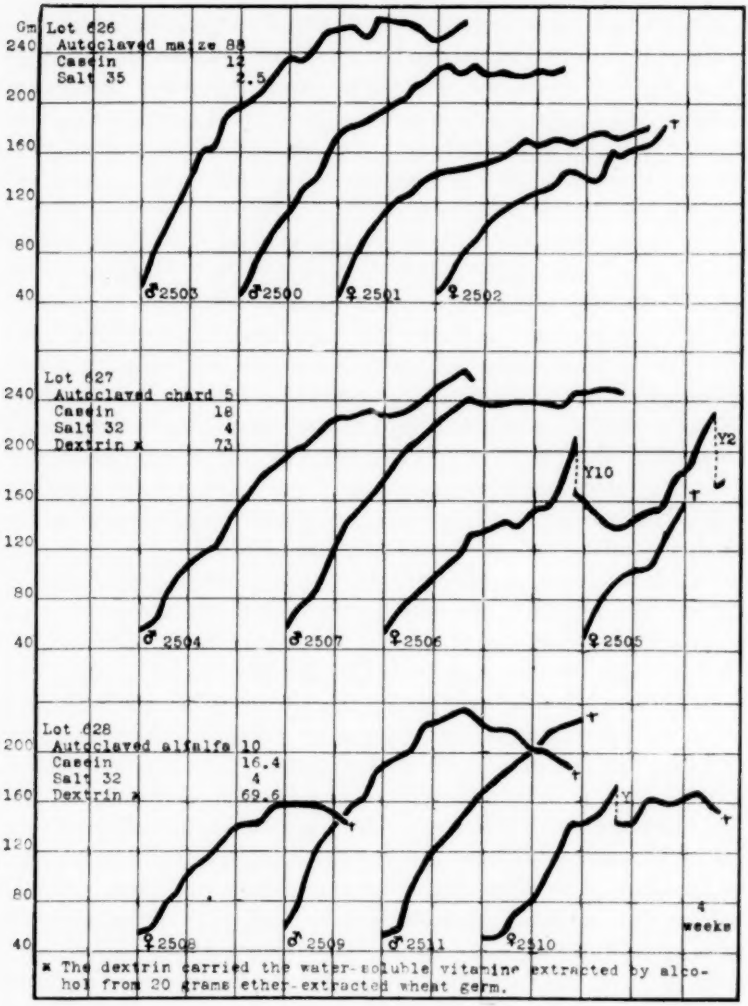
Gm
240
200
160
120
80
40
Lot 626
Autoclaved maize 88
Casein 12
Salt 35 2.5
♂2503
♂2500
♀2501
♀2502
Lot 627
Autoclaved chard 5
Casein 18
Salt 32 4
Dextrin * 73
Y10
Y2
♂2504
♂2507
♀2506
♀2505
Lot 628
Autoclaved alfalfa 10
Casein 16.4
Salt 32 4
Dextrin * 69.6
Y
♀2508
♂2509
♂2511
♀2510
4 weeks
* The dextrin carried the water-soluble vitamine extracted by alcohol from 20 grams ether-extracted wheat germ.

CHART 1.

diminution of its vitamine content as evident from the remarkable performance of the rats in Lot 627, Rat 2506 even rearing two out of its first litter when reduced to five young. If comparisons are made in the curves of growth of rats in this lot with those in the previous publication (1) where the unheated chard was fed, it will be noted that the latter are more nearly normal. It is believed that this discrepancy can be accounted for by the fact that the rats getting the unheated chard had water-soluble vitamine introduced in their rations as ether-extracted wheat embryo which is more depressing in its effect on growth than an alcoholic extract of the same.

In the experiment designed to bring out the effect of heat on alfalfa the autoclaved alfalfa was fed at a level at least twice as high as necessary for normal growth so that a low degree of vitamine destruction would have remained undetected. As it was, the rats grew normally on this ration for 3 to 4 weeks indicating a sufficiency of the vitamine and then declined and died. Rats 2508 and 2509, and possibly 2510, as further indications of a fat-soluble vitamine deficiency, contracted xerophthalmia before death. The condition of Rat 2511 indicated respiratory difficulty, but was not further examined. It hardly appears justifiable to conclude that a fat-soluble vitamine destruction by heat alone was responsible for this condition, especially in view of the excellent growth that had prevailed up to the time of the incidence of the disease. We reserve drawing final conclusions as either one of two possibilities appears acceptable. Either the rapid decline of the animals was occasioned by infection with an especially virulent form of the organism or organisms responsible for xerophthalmia or else while the fat-soluble vitamine was not destroyed by the heat treatment itself it was made more susceptible to destruction—possibly by liberation from combinations—by the agents operative in the aging process. What these are we do not know, but it is to be remembered that the alfalfa used in these experiments was a commercial alfalfa meal of which the history with respect to method of curing was not known. In one series of experiments carried out 2 years ago we found that the vitamine was not destroyed in the ensiling process. When green immature alfalfa was kept in a 20 gallon tub sealed air tight a considerable temperature and acidity developed in the course of 7 days. At

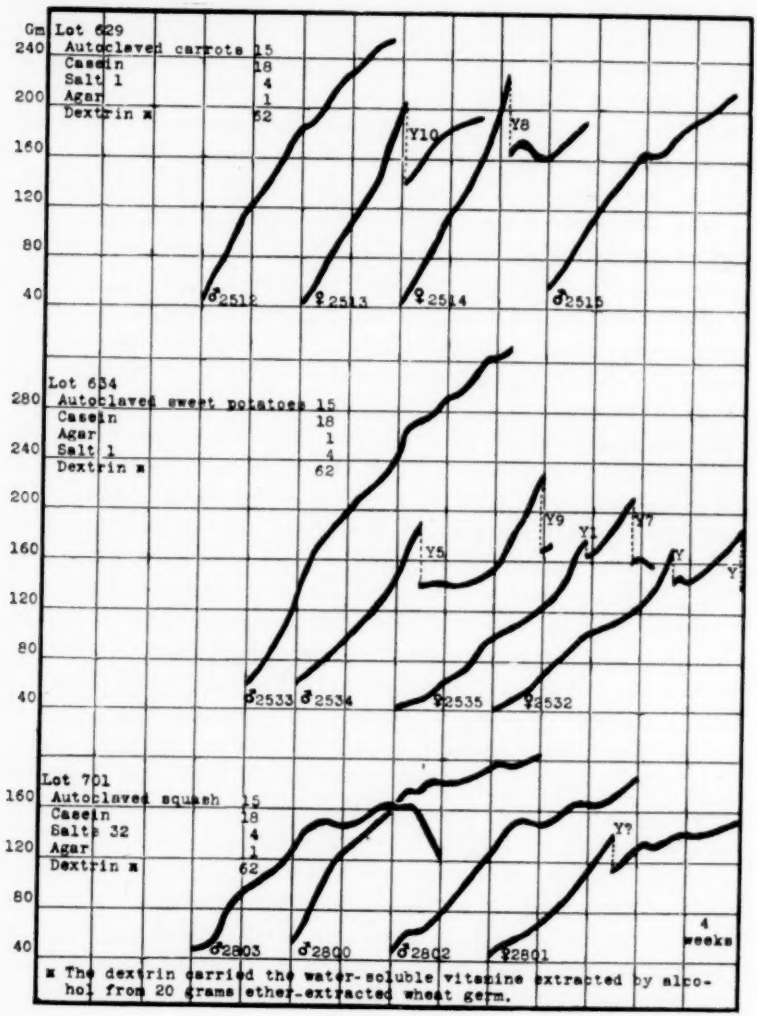
Gm
240
200
160
120
80
40
Lot 629
Autoclaved carrots 15
Casein 18
Salt 1 4
Agar 1
Dextrin x 62
♂2512
♀2513
Y10
♀2514
Y8
♂2515
Lot 634
Autoclaved sweet potatoes 15
Casein 18
Agar 1
Salt 1 4
Dextrin x 62
280
240
200
160
120
80
40
♂2533
♂2534
Y5
♀2535
Y9
Y1
Y7
Y
♀2532
Lot 701
Autoclaved squash 15
Casein 18
Salts 32 4
Agar 1
Dextrin x 62
160
120
80
40
♂2803
♂2800
♂2802
Y?
♀2801
4 weeks
x The dextrin carried the water-soluble vitamine extracted by alcohol from 20 grams ether-extracted wheat germ.

CHART 2.

the conclusion of this period the material when air-dried and fed at a 10 per cent level as the source of both the fat- and water-soluble vitamine allowed a 40 gm. male rat to attain a weight of 240 gm. in 16 weeks. Here we had a considerable acidity acting at a temperature of approximately 35° in the absence of oxygen and later—in the air drying process at room temperature—in the presence of oxygen with no complete destruction of either vitamine.

Carrots and sweet potatoes (9) both excellent sources of the fat-soluble vitamine suffer no appreciable loss of their vitamine by being autoclaved at 15 pounds pressure for 3 hours and then dried at room temperature. This is shown in Chart 2, Lots 629 and 634, where rats made very satisfactory growth when fed with

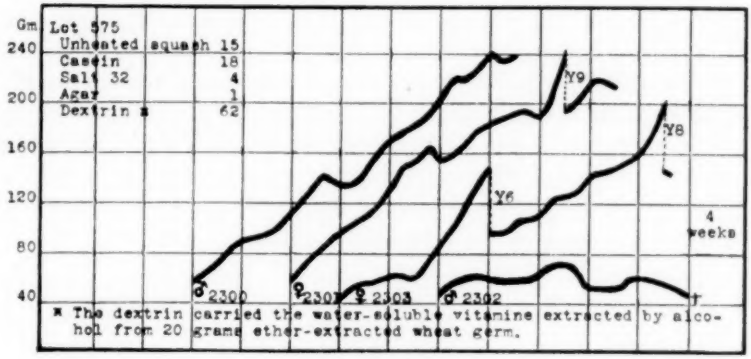

CHART 3.

these materials at 15 per cent levels. In the previous publication a fairly satisfactory degree of growth was reported with the unheated material. That the performance was here improved by the heating process is to be attributed to the reduction of the tendency to tympanites induced by the hemicelluloses in the raw materials. On both heated carrots and sweet potatoes young were successfully reared; Rat 2514, receiving carrots, raising five out of her litter of eight to an average weight of 44 gm. in 4½ weeks and Rat 2534, on sweet potatoes, raising a litter of five to an average weight of 49 gm. in 6 weeks. In view of the performance of the rats on the unheated materials we do not believe that this would have been possible if there had been any considerable destruction of the fat-soluble vitamine.

Up to the present there have not been available any data on the vitamine content of squash. If our theory of the association of certain yellow pigments and the fat-soluble vitamine (10, 11) is generally applicable in the plant kingdom it was to be surmised that the Hubbard squash which has flesh of a golden yellow would be rich in this constituent. We have not yet tested other varieties, but this particular variety was found to contain considerable amounts of the vitamine (Chart 3, Lot 575). Furthermore, like the other materials tested, its vitamine was not destroyed by autoclaving at 15 pounds pressure for 3 hours (Chart 2, Lot 701). The squash used in these experiments had been prepared, after peeling, by drying at room temperature in an air current and later over anhydrous calcium chloride. For autoclaving, it was soaked in water and after autoclaving it was again dried at room temperature. With neither the raw nor the heated squash was the growth entirely satisfactory, but that this was not due to a vitamine deficiency is attested to by the fact that in Lot 575 Rat 2303 successfully reared five young out of her litter of six to an average weight of 49 gm. in 7 weeks. Though this rate of growth was decidedly subnormal they were very active and of good appearance which would not have been the case if there had been a deficiency of the fat-soluble vitamine.

SUMMARY.

A process of heat treatment consisting of autoclaving for 3 hours at 15 pounds pressure does not destroy any of the fat-soluble vitamine as found in yellow maize. Neither does this treatment cause any noticeable destruction of the vitamine in chard, carrots, sweet potatoes, and squash as demonstrated when these materials are fed in percentages of the ration varying from 5 to 15. If some destruction occurred it was not detected, but with the amounts fed it is not believed that it could have occurred to any considerable degree, otherwise normal growth or long continued growth with reproduction would not have been possible. In the case of alfalfa the data are not decisive, but it appears that the autoclaving process in itself did not destroy the vitamine to the extent that a deficiency in the ration was thereby induced. Neither in a short time experiment was any deleterious action

of the ensiling process on the fat-soluble vitamine demonstrable. Our experiments demonstrate that the fat-soluble vitamine as found in the plant kingdom in a grain, in leaf and stem tissue, in fleshy roots, and in a cucurbitous vegetable is comparatively stable at a high temperature.

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PHOSPHORUS REQUIREMENT OF MAINTENANCE IN MAN.*

By H. C. SHERMAN.

With the Cooperation of A. R. Rose, Mathilde Koch, Elizabeth Mathews, and Emil Osterberg.

(From the Department of Chemistry, Columbia University, New York.)

(Received for publication, December 15, 1919.)

It has for some years been inferred from the observations upon intake and output, and recently demonstrated conclusively by feeding experiments with rations otherwise adequate but deficient in one or more of the inorganic elements,[1] that considerable amounts of phosphorus are required in the normal nutrition of animals. Less emphasis than formerly is now placed upon the form of chemical combination in which the phosphorus is furnished by the food. Thus it becomes logical to discuss the amounts of phosphorus required for nutrition under different conditions without necessarily giving detailed consideration to the nature of the food intake in each case. Special interest attaches to the phosphorus requirement in man because the practise of so milling the cereal grains that the phosphorus-rich portions go to the feeding of farm animals while the parts poor in phosphorus are used for human food makes the danger of phosphorus deficiency relatively greater in human than in animal nutrition, unless it is found by adequate quantitative investigation that this tendency is fully offset by certain factors of safety of which two may be especially mentioned: (1) Growth is relatively less rapid in the child than in the young of most domesticated animals, while muscular activity at corresponding ages is probably fully as pronounced; (2) adult man may in the majority of cases be somewhat more active than the average of those adult farm animals which are

* Published as contribution No. 331 from the Department of Chemistry, Columbia University.

[1] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxiv, 131.

kept merely in a condition of maintenance or fattening as distinguished from those which serve as draft animals. For either or both of these reasons it may be that the ratio of phosphorus requirement to energy requirement is enough lower in man than in his farm animals to counterbalance the tendency to a lower percentage of phosphorus in his food, as long as his total food intake is adequate to meet his energy requirement. That grain products may constitute a smaller proportion of the entire food intake of man than of many farm animals may or may not be a factor of safety—for, while some foods such as milk and eggs are very effective supplements to the grain products in this respect (as in several others), other foods equally widely used such as butter and sugar are practically devoid of phosphorus compounds so that their inclusion in the dietary puts man at a further disadvantage in respect to his phosphorus intake.

During the past 15 years a considerable number of experiments upon the phosphorus requirement of maintenance in man have been carried out in this laboratory. The data of several of these have been included in papers previously published, sometimes with,[2] sometimes without,[3] specific discussion of their bearing upon the present problem. The purpose of this paper is to place on record such of our experimental data as have not appeared elsewhere, and to summarize briefly the evidence of all available experiments which now seem to us to permit of direct quantitative comparison.

Experimental Data Not Recorded in Previous Papers.

Subject F, a woman of 64 kilos (studied in 1911), on a diet of bread, meat, potato, rice, prunes, butter, sugar, and tea which was estimated to furnish 0.95 gm. of phosphorus per day, showed the following average elimination in four successive periods of 3 days each which followed each other without intermission: in urine, 0.55, 0.53, 0.45, 0.50; in feces, 0.27, 0.30, 0.19, 0.23 gm.

[2] Sherman, H. C., Mettler, A. J., and Sinclair, J. E., *U. S. Dept. Agric., Office Exp. Stations, Bull. 227*, 1910. Sherman, H. C., Gillett, L. H., and Pope, H. M., *J. Biol. Chem.*, 1918, xxxiv, 373. Sherman, H. C., Wheeler, L., and Yates, A. B., *J. Biol. Chem.*, 1918, xxxiv, 383.

[3] Sherman, H. C., and Winters, J. C., *J. Biol. Chem.*, 1918, xxxv, 307.

respectively. It is possible that the actual intake may have been somewhat less than the analytical data indicate due to an accident which permitted partial drying of some of the foods while the samples were being taken for analysis. This, however, would in no wise affect the data of the output which we take to be an approximate indication of the phosphorus requirement of this subject (see Nos. 21 to 24 in Table I).

Subject I, a man of 61 kilos studied by A. R. Rose and the writer in 1912, took a diet consisting of bread, wheat farina, rice, egg white, milk, butter, coffee, and apple sauce, giving an intake in five successive experiments of 0.65, 0.65, 0.64, 0.56, and 0.56 gm. of phosphorus per day. These experiments followed each other without intermission and were preceded by 3 days of similar diet. The average daily output of phosphorus in each of the five experiments was as follows: in urine, 0.57, 0.60, 0.56, 0.46, 0.43; in feces, 0.26, 0.12, 0.14, 0.16, 0.09 gm. respectively. There was thus a slight minus balance at first and practical equilibrium in the latter part of the time covered by the investigation. The data of output calculated to uniform basis appear under Nos. 25 to 29 in Table I.

Subject H, a woman of 65 kilos studied in 1914, took a diet of bread, butter, wheat breakfast food, meat, potato, peanuts, and oranges with an intake of 0.61 gm. of phosphorus per day and an average daily output in each of three successive 3 day experiments as follows: in urine, 0.60, 0.68, 0.76; in feces, 0.17, 0.17, 0.19 gm. respectively (Nos. 30 to 32 in Table I).

Subject E, a man of 69 kilos studied in 1916, after 10 days on phosphorus-poor food, took during a 3 day experiment a diet of bread, butter, meat, apple, and milk furnishing 0.76 gm. of phosphorus, while the daily output for the same 3 day period was: in urine, 0.75; in feces, 0.18 gm. (No. 71 in Table I).

Subject R, a man of 80 kilos (1916), took during a period of 15 days in five experiments of 3 days each, a diet of bread, butter, and apples which furnished in the successive periods 0.56, 0.53, 0.49, 0.52, 0.57 gm. of phosphorus per day; the average daily output during each experiment was: in urine, 0.61, 0.46, 0.51, 0.43, 0.44; in feces, 0.19, 0.16, 0.19, 0.19, 0.15 gm. respectively (Nos. 72 to 76 in Table I).

TABLE I.*

Indicated Phosphorus Requirements for Maintenance per 70 Kilos of Body Weight per Day.

Experiment No.	Phosphorus.	Experiment. No.	Phosphorus.	Experiment No.	Phosphorus.
	gm.		*gm.*		*gm.*
1	0.87	33†	0.84	65†	1.04
2	0.95	34†	0.85	66†	0.80
3	0.83	35†	0.71	67†	0.89
4	1.02	36†	0.74	68†	0.89
5	1.09	37†	0.74	69†	0.89
6	0.78	38†	0.69	70†	0.98
7	0.89	39†	0.76	71	0.95
8	0.68	40†	0.76	72	0.70
9	0.73	41†	0.68	73	0.54
10	1.06	42†	0.90	74	0.62
11	1.13	43†	0.89	75	0.54
12	1.12	44†	1.14	76	0.52
13	0.98	45†	1.01	77†	0.85
14	1.19	46†	1.01	78†	0.88
15	1.04	47†	0.95	79†	0.78
16	0.90	48†	1.07	80†	0.76
17	0.96	49†	1.04	81†	0.82
18	1.13	50†	1.03	82†	0.88
19	1.04	51†	0.85	83†	0.89
20	1.02	52†	0.94	84	0.77
21†	0.90	53†	0.91	85	0.79
22†	0.91	54†	0.91	86	0.69
23†	0.70	55†	0.88	87†	0.72
24†	0.80	56†	0.90	88†	0.74
25	0.96	57†	1.06	89†	0.75
26	0.83	58†	1.01	90†	0.65
27	0.81	59†	1.01	91†	0.72
28	0.72	60†	1.03	92†	0.80
29	0.60	61†	0.90	93†	0.81
30†	0.83	62†	1.08	94†	1.20
31†	0.93	63†	1.07	95†	1.17
32†	1.03	64†	0.90		
Average.....					0.88

* Experiment 1, Sivén, V. O., *Skand. Arch. Physiol.*, 1901, xi, 308. Nos. 2 and 3, Gumpert, E., *Med. Klin.*, 1905, i, 1037. Nos. 4 and 5, Sherman, H. C., Mettler, A. J., and Sinclair, J. E., *U. S. Dept. Agric., Office of Exp. Stations, Bull. 227*, 1910. Nos. 6 to 9, Hämäläinen, J., and Helme, W.,

Subject Q, a man of 68 kilos (1917), took a diet of bread and milk which furnished in three successive 4 day experiments daily intakes of 0.70, 0.51, and 0.53 gm. of phosphorus respectively. The corresponding data of output were: in urine 0.58, 0.53, 0.53; in feces 0.20, 0.24, 0.12 gm. of phosphorus per day (Nos. 84 to 86 in Table I).

General Comparison of Available Data.

For convenience of comparison and discussion the data of these experiments and of all available previously published experiments which seem to be quantitatively comparable, have been calculated to the uniform basis of phosphorus output per day per 70 kilos of body weight on the same general principle as in the similar study of the protein requirement of maintenance.[4] The data of "indicated phosphorus requirement" thus found are summarized in Table I.

It will be seen that the data of the 95 experiments range from a minimum of 0.52 to a maximum of 1.20 gm. with an average of 0.88 gm. of phosphorus per 70 kilos of body weight per day. The experiments upon men average 0.87 gm. and those upon women average 0.89 gm. per 70 kilos per day. The range of variation among the experiments is here quite similar (proportionately) to that found in a corresponding compilation of ex-

Skand. Arch. Physiol., 1907, xix, 182. Nos. 10 to 13, Berg, R., *Biochem. Z.*, 1911, xxx, 107. Nos. 14 to 20, Aron, H., and Hocson, F., *Biochem. Z.*, 1911, xxxii, 189. Nos. 21 to 24, Koch, M., and Sherman, H. C., not previously published. Nos. 25 to 29, Rose, A. R., and Sherman, H. C., not previously published. Nos. 30 to 32, Sherman, H. C., and Mathews, E. M., not previously published. Nos. 33 to 50, Sherman, H. C., Gillett, L. H., and Pope, H. M., *J. Biol. Chem.*, 1918, xxxiv, 373. Nos. 51 to 63, Sherman, H. C., Wheeler, L., and Yates, A. B., *J. Biol. Chem.*, 1918, xxxiv, 383. Nos. 64 to 70, Sherman, H. C., Gillett, L. H., and Pope, H. M., *J. Biol. Chem.*, 1918, xxxiv, 373. No. 71, Sherman, H. C., not previously published. Nos. 72 to 76, Sherman, H. C., and Osterberg, E., not previously published. Nos. 77 to 83, Sherman, H. C., Wheeler, L., and Yates, A. B., *J. Biol. Chem.*, 1918, xxxiv, 383. Nos. 84 to 86, Sherman, H. C., and Beegle, F. M., not previously published. Nos. 87 to 95, Sherman, H. C., and Winters, J. C., *J. Biol. Chem.*, 1918, xxxv, 307.

† Experiments upon women.

[4] Sherman, H. C., *J. Biol. Chem.*, 1920, xli, 97.

periments upon the protein requirement. The same causes of variation are doubtless operative, but to different degrees in the two cases. In the study of protein requirement the earlier experiments in general tended to give high results because of failure to plan the diets properly or to continue them for a long enough time. These errors probably play less part in the phosphorus experiments here quoted since they are in the main of recent date and have been planned and carried out with these considerations in mind. As the experiments are arranged chronologically in Table I, it may be seen at a glance that there is no marked tendency to lower results among the later experiments. Probably, therefore, the question whether or not the experiment was sufficiently controlled and continued for a long enough time to test actual requirements was a less disturbing factor here than in the study of protein requirement. The function of phosphates in the maintenance of neutrality in the body may be one reason for the variations in the phosphorus output, although it has been shown[5] that the surplus acid arising from the normal metabolism of a diet in which the acid-forming elements predominate may be eliminated in part as ammonia salt and in part as increased acidity of urine without necessarily increasing the output of phosphorus.

It is possible too that the nature of the phosphorus compounds of the intake may be of more significance than has usually been assumed during recent years. The fact that inorganic forms of phosphorus may serve to meet all the requirements of the phosphorus metabolism does not necessarily imply that all the phosphorus compounds of the intake have exactly the same quantitative efficiency in nutrition, when supplied in amounts barely adequate to meet actual needs.

While several of the factors determining the phosphorus output remain for further quantitative investigation, we are probably justified in concluding that we now know the phosphorus requirement with about the same probable accuracy that the protein requirement is known, and that about one-fortieth to one-fiftieth as much phosphorus (reckoned as element) as of protein is required in the maintenance metabolism of man.

[5] Sherman, H. C., and Gettler, A. O., *J. Biol. Chem.*, 1912, xi, 323.

Compared with the quantities actually required for maintenance, the average food intake of typical American households appears to provide a somewhat more liberal margin of protein than of phosphorus. Yet in a detailed study of the food supplies of 224 families or other groups of people selected as typical of the population of different parts of the United States only eight showed less than 0.88 gm. of phosphorus per man per day and in all but two of these cases the phosphorus content would have reached this figure if the food consumed (without change in its character) had been increased in amount to a level of 3,000 calories per man per day. The two cases which apparently contained less than the average actual requirement of phosphorus and would still have been thus deficient if the food had been sufficient in amount to cover the energy requirement amply were both reported from southern states. McCollum, Simmonds, and Parsons, in discussing the results of experiments upon rats with diets made up in imitation of those whose use has been described as resulting in pellagra in man, express the opinion that "it may well be that the preponderance of products of the endosperm of seeds made the phosphorus content of the diet too low."[6] This is in addition to the inadequacy of the food as regards calcium, fat-soluble A, and certain amino-acids, which they regard as characterizing the "pellagra-producing" diets of the South. Outside of the southern regions where the food supply consists too largely of patent flour or new process (degerminated) corn-meal supplemented chiefly by sugars and fats, the danger that a freely chosen American dietary will be deficient in either protein or phosphorus does not appear serious, in the light of our present evidence, so far as the requirements of maintenance are concerned.

What quantities of phosphorus in the food will best meet the requirements of growth, pregnancy, and lactation remains to be determined.

[6] McCollum, E. V., Simmonds, N., and Parsons, H. T., *J. Biol. Chem.*, 1919, xxxviii, 130.

THE RÔLE OF PENTOSE-FERMENTING BACTERIA IN THE PRODUCTION OF CORN SILAGE.*

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(Received for publication, December 19, 1919.)

In a previous publication[1] on the xylose-fermenting bacteria, it was noted that these organisms are easily isolated from silage, and apparently occur there in large numbers. Their optimum temperature for fermentation was shown to be about 27°C., which is approximately the average temperature found in ensiled corn. In relation to oxygen supply the pentose fermenters are most active when subjected to a low oxygen tension and will ferment xylose under anaerobic conditions. In this respect the reduced oxygen supply usually found in a silo should furnish suitable conditions for their growth.

The pentose fermenters are particularly characterized by the ease and rapidity with which they ferment pentoses, producing acetic acid and lactic acid as the chief end-products. Unpublished data on their power to ferment other sugars led to the interesting observation that from glucose they formed large quantities of alcohol, lactic acid, carbon dioxide, and small quantities of acetic acid. The fermentation of fructose by these organisms results in the formation of a characteristic product, mannitol. Dox and Plaisance[2] have isolated mannitol from silage in consider-

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[1] Fred, E. B., Peterson, W. H., and Davenport, A., *J. Biol. Chem.*, 1919, xxxix, 347.

[2] Dox, A. W., and Plaisance, C. P., *Iowa Agric. Exp. Station, Research Bull. 42*, 1917.

able quantities and its presence there is generally attributed to bacterial action.

Since xylose, or xylose-yielding substances, glucose, fructose, and other sugars, are found in abundance in green corn and since the above fermentation products are the chief chemical compounds formed in silage, a study of the relationship of these bacteria to silage production was undertaken.

For many years the relationship of microorganisms and of plant enzymes to the fermentation of silage has been studied. Some investigators have concluded that microorganisms are solely responsible for the changes in the ensilage, others that the plant enzymes are the chief causative agent. A third group of investigators has held that both factors are involved in this fermentation.[3]

In this article a brief report is presented of the results obtained from the inoculation of corn fodder at the time it was placed in the silo.

EXPERIMENTAL.

Numerous investigators have shown that normal silage can be produced under laboratory conditions by filling small containers with the cut corn and allowing the ensiled material to stand at a suitable temperature for a short time. The silage thus made possesses the same odor and taste, and yields on analysis the same products as silage made in the usual way. In these experiments, milk bottles closed with a one-hole rubber stopper through which passed a bent glass tube were used. The free end of the glass tube was sealed with mercury by inserting it into a test-tube containing 2 or 3 inches of mercury. Such an arrangement has been used with excellent results by Professor E. G. Hastings of the Agricultural Bacteriology Department in the experimental study of silage.

The first series was packed with rather green corn on September 5, 1919, and when filled contained 350 gm. of material in

[3] For a review of the literature see Dox, A. W., and Neidig, R. E., *Iowa Agric. Exp. Station, Research Bull. 7*, 1912. Neidig, R. E., *Iowa Agric. Exp. Station, Research Bull. 16*, 1914. Sherman, J. M., *J. Bacteriol.*, 1916, i, 452. Lamb, A. R., *J. Agric. Research*, 1917, viii, 378. Hunter, O. W., *J. Agric. Research*, 1917, x, 82.

each bottle. Two bottles were used as controls and, except for the addition of 50 cc. of water, were untreated. Two others were sterilized, 50 cc. of sterilized water added, and kept as sterilized controls. Four bottles were sterilized and subsequently inoculated in duplicate with 50 cc. of a water suspension of the pentose-fermenting bacteria, Cultures 41–11 and 118-8. In order to insure growth, another set of bottles was inoculated with the same organism suspended in yeast water. The addition of this yeast water was found to be unnecessary as the bacteria grew just as well in the bottles to which no yeast water was added. The bottles were then incubated for 10 days at 27°C. During the period of incubation a strong evolution of gas was noted in all the bottles except in the sterilized uninoculated controls. In these a negative pressure was indicated by the mercury rising in the glass tube. When the bottles were opened, all except the sterilized uninoculated controls had about the same odor and taste as that of a normal silage. The contents of each bottle were put through a meat chopper to insure a uniform material, a sample was taken for the moisture determination, and as much juice expressed from the remainder as could be obtained with a strong hand press. This juice was analyzed for volatile and non-volatile acids by the usual methods; *viz.*, steam distillation for volatile acids and ether extraction of the residue from the steam distillation for non-volatile acids. The treatment of the corn fodder together with the results of the analyses is given in Table I.

An examination of the results of this table shows that in the unsterilized group there is a large production of acids and alcohol. A comparison of the products formed in the unsterilized silage with and without pentose fermenters added shows that the addition of these organisms caused a noticeable increase in volatile acid, non-volatile acid, and alcohol. These bacteria in the presence of the microorganisms commonly found in silage were able to bring about well defined differences in the chemical composition of silage.

In the sterilized group the action of these organisms is much more striking than in the raw and uninoculated group. In the absence of plant enzymes and microorganisms these bacteria brought about a decided increase in volatile and non-volatile

acid, and also produced a small amount of alcohol. When compared with the products naturally present in the sterilized, uninoculated silage, it is evident that the pentose-fermenting bacteria are very active in fermentation of sterilized corn fodder. It is of particular interest that these bacteria in the absence of other forms are able to produce in large amounts the chief substances which are characteristic of silage. From the data, it

TABLE I.

Analysis of Silage Formed from Corn with and without Inoculation.

Treatment.	100 gm. of dry silage.			
	Moisture.	Volatile acid as acetic.	Non-volatile acid as lactic.	Alcohol as ethyl alcohol.
	per cent	*gm.*	*gm.*	*gm.*
Untreated corn.	80.4	0.684	6.253	2.007
Unsterilized and inoculated with Culture 41–11.	81.1	1.502	7.357	2.631
Unsterilized and inoculated with Culture 118–8.	80.4	1.480	8.389	2.552
Sterilized control.	76.2	0.240*	1.999*	
" inoculated with Culture 41–11.	78.1	1.476	4.598	0.557
" " " " 118–8.	77.2	1.207	4.387	0.311
Sterilized control plus yeast water.	76.5	0.255	1.474	
" plus yeast water inoculated with Culture 41–11.	78.1	1.370	3.454	0.543
Sterilized, plus yeast water inoculated with Culture 118–8.	78.8	1.495	4.748	0.736

* These high values are no doubt due largely to chemical changes incident to the high temperature and length of time of sterilization.

appears that the pentose fermenters either in the presence or in the absence of other microorganisms are capable of bringing about an acid fermentation of silage which is comparable with that of normal silage.

From the data for the third group, sterilized corn plus yeast water and bacteria, it will be noted that the yeast water has little if any effect on fermentation.

On September 25, another series of bottles was set up in order to study the effect of inoculation by other acid-producing bacteria

as well as the pentose fermenters. The organisms chosen were *Bacillus lactis acidi* and *Bacillus bulgaricus*. The quantity of carbon dioxide produced by the pentose fermenters was determined in this series. This was absorbed in strong alkali and the latter analyzed by means of the Van Slyke apparatus for the determination of carbon dioxide in blood and in other carbonate solutions. The results are given in Table II.

TABLE II.

Effect of Inoculation with Different Types of Acid-Producing Bacteria.

Treatment.	100 gm. of dry silage.				
	Moisture.	Volatile acid as acetic.	Non-volatile acid as lactic.	Ethyl alcohol.	Carbon dioxide.
	per cent	*gm.*	*gm.*	*gm.*	*gm.*
Untreated corn.	74.6	2.436	2.844	0.908	Not determined.
Sterilized and uninoculated.	72.9	0.352	2.002	None.	" "
Unsterilized and inoculated with *B. lactis acidi.*	67.0	1.033	2.862	0.966	" "
Sterilized and inoculated with *B. lactis acidi.*	70.8	0.339	2.305	None.	" "
Unsterilized and inoculated with *B. bulgaricus.*	70.3	1.231	3.007	"	" "
Sterilized and inoculated with *B. bulgaricus.*	68.6	0.493	Lost.	"	" "
Sterilized and inoculated with pentose fermenter No. 41–11.	71.9	1.562	3.801	0.568	0.876
Sterilized and inoculated with pentose fermenter No. 118–8.	73.0	1.335	3.475	Lost.	1.088

A study of the data of Table II clearly reveals the superiority of the pentose fermenters as compared with the other two types of bacteria in the formation of the substances usually found in silage. The latter produced little or no increase over the controls while the influence of the former is manifest in every case where they were present.

Carbon dioxide is shown to be produced by the pentose bacteria in large quantities but no comparison with the other organisms in this respect can be made as the quantity of gas evolved from these cultures was not determined.

SUMMARY.

The results of the experiments reported in this paper indicate that the pentose-fermenting bacteria are capable of bringing about decided changes in raw or in sterilized corn tissue. When added to raw corn fodder, these organisms are able to compete with the fermentation processes which normally occur. In sterilized silage the pentose fermenters develop rapidly and produce the substances commonly found in good silage; *viz.*, acetic acid, lactic acid, ethyl alcohol, and carbon dioxide.

From the standpoint of temperature, oxygen supply, and fermentable compounds, silage offers a suitable medium for the growth of the pentose fermenters. The authors feel that the results indicate that these bacteria play an important part in the formation of corn silage.

NOTE ON THE HYDROGEN ION CONCENTRATION OF THE HUMAN DUODENUM.

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(Received for publication, December 29, 1919.)

It has long been known that the stomach is acid in comparison to the ileum, and it follows from this that the reaction of the duodenum must be influenced by the opening of the pylorus. According to Cannon, the opening of the pylorus is controlled by the acidity of this region, but it has been shown by a number of workers, most recently by Luckhardt, Phillips, and Carlson, that the motor phenomena of the stomach have a great influence on the passage of its contents through the pylorus. Perhaps the following description is nearly correct: Acid influences the tone of the pylorus but whenever this is less than the tone of the stomach or duodenum the passage of fluid may occur. The passage must necessarily be toward the region of lesser tone and is not always in the same direction, as shown by the fact, known for a long time, that the stomach contents may occasionally be bile-stained. Since the chyme is acid and the pancreatic juice alkaline, the relative rate of the flow of these two into the duodenum must influence its reaction.

In 1915 Dr. John P. Schneider gave one of us two samples of human duodenal contents which were found to be of pH 1.5 and 7.61. He had been removing the duodenal contents from many of his patients with an Einhorn duodenal tube in order to estimate the bile pigments. He allowed the duodenal contents to syphon out of the tube and found that the flow was intermittent. Sometimes the fluid spurted out and it was then acid to litmus whereas otherwise it was not acid to litmus (paper). It was shown by McClendon that the duodenum of the infant, although always acid, was very variable, the reaction sometimes approach-

ing neutrality (pH = 6.3). Long and Fenger, using the duodenal tube, observed great variation in the adult duodenum (pH = 3.80 to 7.81). The technique used in the present paper differs from theirs only in the use of the hydrogen electrode described by McClendon and Magoon.

The Einhorn duodenal tube was swallowed by one of us (F.J.M.) and allowed to descend to the first mark, then carefully lowered to the second mark. The subject was then placed on his right side on a couch, with his hips elevated. An average of about 3.5 hours was required before the end of the tube with the lead

TABLE I.

Meal.	Food taken.	pH
Breakfast.	Hot cakes, toast, coffee.	3.80
"	Bacon, rice, coffee.	3.20
"	Corn flakes and cream, eggs, toast.	6.98
Luncheon.	Beef, potatoes, tomatoes, pie.	4.60
"	Pork, " eggplant, cake.	5.00
Breakfast.	Hot cakes, pineapple sauce, coffee.	7.21
"	Toast, raspberry sauce.	7.40
"	Ham, eggs, toast, coffee.	7.54
Luncheon.	Beef, potatoes, corn, ice-cream.	7.00
"	" " bread, melon, iced tea.	7.82
"	Potatoes, carrots, pie, cake, ice-cream, milk.	7.60
"*	Beef, onions, potatoes, pie, cake followed by bismuth.	7.54

* The fluoroscope was used in conjunction with bismuth and the duodenal tube after the digestion of the food was well under way.

weight seemed to be in the duodenum. The degree of traction on the tube was at first used as an index of its passage of the pylorus, but since some of the samples were acid we began to doubt that the pylorus had been passed, and confirmed this by use of the fluoroscope, for which our thanks are due the University Hospital.

In Table I the determinations that were acid and those that were alkaline are separated for comparison. We have no doubts that the acid samples came from the duodenum, since all samples were taken about 3 or 4 hours after the last meal and it has been shown by McClendon that the pH of the stomach at this time was between 1 and 2.5 in all normal individuals examined, whereas

the acid samples reported in Table I have a pH between 3.2 and 6.98.

After the position of the lead weight on the end of the duodenal tube in the duodenum was shown by means of the fluoroscope, it

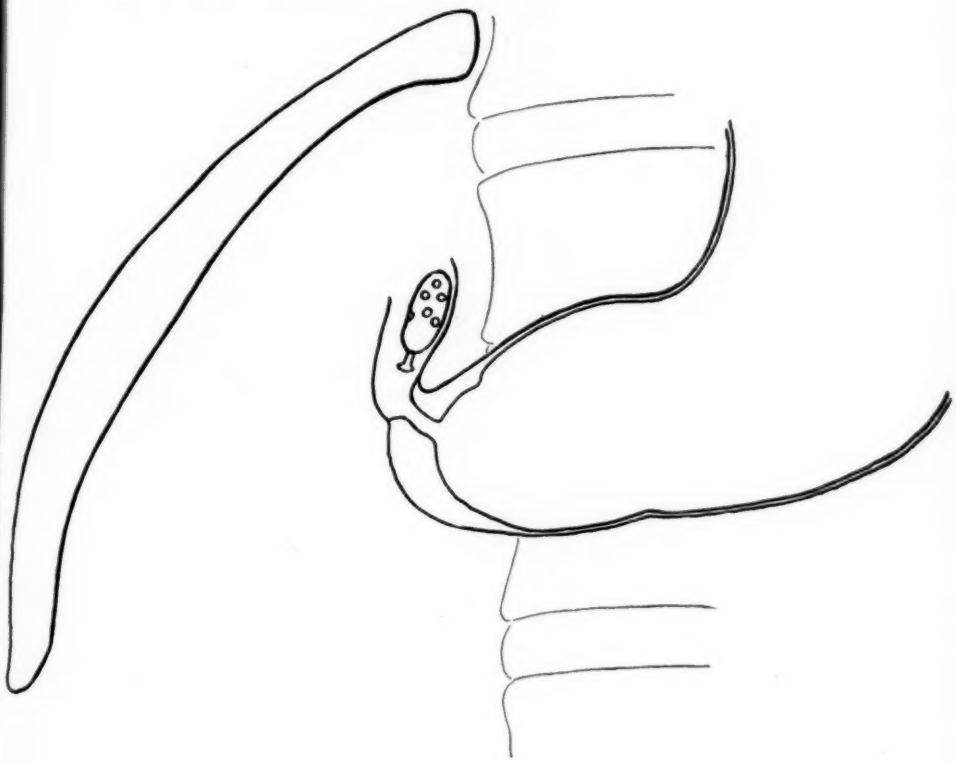

FIG. 1.

was drawn down toward the pylorus by traction on the tube and a photograph taken. A tracing from the x-ray plate is shown in Fig. 1. The dim edges of shadows in the plate were sharpened by free-hand drawing in making the tracing.

CONCLUSIONS.

The reaction of the duodenum between 3 and 4 hours after meals was usually found to fluctuate around the neutral point, but the extreme range on the acid side was greater than on the alkaline side, possibly due to the spurting of gastric contents into the duodenum.

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STUDIES OF ACIDOSIS.

XV. CARBON DIOXIDE CONTENT AND CAPACITY IN ARTERIAL AND VENOUS BLOOD PLASMA.

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(Received for publication, December 29, 1919.)

According to the facts at our disposal, the bicarbonate content of the arterial blood plasma appears to be the blood figure most accurately indicating the alkaline reserve of the body fluids as a whole (Van Slyke and Cullen, 1917; Palmer and Van Slyke, 1917). In man it has heretofore been necessary to depend upon bicarbonate determinations in the venous blood as the closest practicable approximation to the arterial bicarbonate. In practice this has been estimated by determining the carbon dioxide capacity; that is, the CO_2 content of the plasma after saturation with air containing CO_2 at approximately the tension of normal alveolar air (Van Slyke and Cullen, 1917). The reliability of such determinations for the diagnosis of acidosis in metabolic diseases may be considered as demonstrated by the tests to which the method has been put in various laboratories, but it nevertheless remains desirable to compare the results thus obtained on the venous plasma with the actual arterial bicarbonate. The utilization of a technique for arterial punctures (Stadie, 1919) has rendered it possible to make this comparison in a series of patients, and the results are presented in this paper. Altogether thirty individuals were studied, most of whom had bronchopneumonia or lobar pneumonia of varying degrees of severity. A few normal individuals are included.

Methods.

The arterial blood was obtained as previously outlined (Stadie, 1919). The venous blood was taken without stasis, and, as a rule, 1 to 3 minutes after the arterial; both arterial and venous bloods were collected out of contact with air under albolene.

The CO_2 content and capacity were determined by the methods of Van Slyke (1917) and Van Slyke and Cullen (1917) respectively. The blood, collected under albolene to prevent loss or gain of CO_2, was centrifuged, and 1 cc. samples of the plasma were withdrawn and discharged into the cup of the Van Slyke apparatus under a little ammonia to prevent escape of CO_2. After thus determining the CO_2 content, the remaining plasma was saturated with air containing approximately 5.5 volumes per cent of CO_2, and the CO_2 capacity then determined.

In calculating CO_2 *capacity* (by Table I, Van Slyke and Cullen, 1917) the CO_2 physically dissolved (H_2CO_3) is subtracted, so that the results represent only CO_2 bound as bicarbonate.

In calculating the CO_2 content, however (by Table I, Van Slyke, 1917), no subtraction for physically dissolved CO_2 is made, and the data represent total CO_2 from $NaHCO_3$ and H_2CO_3 together. The free CO_2 in normal arterial plasma is about 3 volumes per cent; that is, a CO_2 content of 65 volumes per cent represents approximately 62 volumes per cent of bicarbonate CO_2 and 3 volumes per cent of free carbonic acid CO_2.

The arterial oxygen unsaturation, or the percentage of hemoglobin in the arterial blood not combined with oxygen, was calculated as described by Lundsgaard (1918) using Van Slyke's method (1918) for the oxygen determinations. The oxygen content of the arterial blood was determined, and then a portion was thoroughly aerated, and the oxygen capacity determined.

$$\text{Per cent oxygen unsaturation} = \frac{\text{O}_2\text{ capacity} - \text{O}_2\text{ content}}{\text{O}_2\text{ capacity}} \times 100.$$

In normal individuals at rest the arterial unsaturation averages about 5 per cent, 95 per cent of the hemoglobin in the arterial blood being saturated with oxygen. Figures for the unsaturation higher than 8 per cent indicate incomplete oxygenation of the arterial blood.

The results are given in Table I. In a few cases the arterial CO_2 content equals or slightly exceeds the venous. This may have been due to the short differences in time between the drawing of arterial and venous bloods, or to a summation of experimental errors in the two determinations. The latter factor cannot be excluded because in many cases the amount of material did not permit analyses in duplicate. It is believed,

TABLE I.

Case No.	Arterial plasma.		Venous plasma.		A Arterial content / Arterial capacity	B Arterial content / Venous capacity	C Arterial content / Venous content	Arterial oxygen unsaturation.
	CO_2 content.	CO_2 capacity.	CO_2 content.	CO_2 capacity.				
	vol. per cent	*vol. per cent*	*vol. per cent*	*vol. per cent*				*per cent*
3		60.3		60.3				11.9
	65.7	67.2	66.6	70.0	0.978	0.939	0.988	9.0
	69.9		74.6	75.8		0.922	0.937	6.0
	70.8	72.9	71.1	72.9	0.971	0.971	0.996	0
6	65.2		71.8	69.2		0.942	0.942	
8			67.3	73.7				8.1
9	50.3	56.8	50.8	54.9	0.886	0.916	0.990	68.2
10	69.0	73.0	68.4	71.6	0.945	0.964	1.009	4.6
11	57.3	62.4	59.2	64.9	0.918	0.883	0.968	10.1
	62.6	67.5	66.0	68.9	0.928	0.909	0.948	8.9
12	59.5	66.5	63.8	69.4	0.895	0.857	0.933	6.3
	56.2	61.7	60.1	64.6	0.911	0.870	0.935	13.7
13	63.5	66.2	62.5	66.2	0.959	0.959	1.016	2.8
	61.9	62.1	64.7	65.0	0.997	0.952	0.957	7.5
14	63.2	70.0			0.903			20.7
15	55.5	62.3	60.9	66.3	0.891	0.837	0.911	7.8
	63.1	65.3	67.4	69.1	0.966	0.913	0.936	7.3
16	55.8	59.4	59.7	66.7	0.939	0.837	0.935	14.1
17	59.6	62.2	60.5	62.2	0.958	0.958	0.985	16.3
	69.1	71.6	77.6	78.1	0.965	0.885	0.891	11.5
18	56.0	63.3	57.8	63.3	0.885	0.885	0.969	16.6
	61.4	64.0	59.0	64.0	0.959	0.960	1.041	16.5
			63.1	70.8				38.2
19	63.9	68.4	65.8	68.4	0.934	0.934	0.971	7.9
	57.1	76.5	61.8	76.0	0.746	0.751	0.924	0
20	55.7	62.0		61.1	0.898	0.912		13.4
22	63.3	70.8	71.1	75.5	0.894	0.838	0.891	19.5
	46.8	51.9	51.5	50.0	0.902	0.936	0.909	25.1
23	66.8	62.1	69.7	69.6	1.076	0.960	0.959	15.1
			67.2	78.1				8.9
24	64.5	66.2	71.2	68.1	0.974	0.947	0.906	2.4
25			54.9	50.9				44.1
26	63.7		71.9				0.886	5.2
30	50.2	52.6	57.3	65.8	0.954	0.763	0.876	24.9
33	55.5	60.0	58.4	57.2	0.925	0.970	0.950	23.4
34	58.8	61.4			0.958			14.9
	53.3	57.2	53.3	59.2	0.932	0.900	1.000	33.5
	56.7	58.8			0.964			25.9
	56.1	57.8			0.971			10.7
38	56.2		60.6	63.2		0.889	0.927	8.4
39	57.4	61.0	59.3	66.8	0.941	0.859	0.968	14.5
40	59.8	59.5			1.005			
41	55.7	61.0			0.913			
42	50.8	51.4			0.988			
Average.....					0.938	0.904	0.950	

however, that the number of determinations is sufficient to rule out the influence of such factors on the results viewed as a whole. The latter seem to justify the following conclusions.

CONCLUSIONS.

1. The carbon dioxide capacity ($NaHCO_3$) of the venous blood plasma in man determined by the technique of Van Slyke and Cullen (1917) parallels the arterial plasma carbon dioxide content ($NaHCO_3 + H_2CO_3$), which it exceeds on the average by about one-tenth. As the arterial CO_2 is 95 per cent due to bicarbonate, the above results mean that the venous plasma carbon dioxide capacity parallels the arterial plasma bicarbonate, averaging about 115 per cent as great (Column B of Table I).

2. The carbon dioxide *content* of plasma from venous blood drawn without stasis parallels the arterial slightly more closely than does the venous carbon dioxide capacity and averages 105 per cent of the arterial content (Column C).

3. Consequently, for estimating the alkaline reserve in man, the venous plasma may be used for CO_2 determination directly, without resaturation with carbon dioxide, if the blood is centrifuged and the plasma brought to analysis without opportunity for escape of CO_2.

4. Even when pulmonary conditions in pneumonia become so unfavorable for gas exchange that the arterial blood is incompletely oxygenated (high oxygen unsaturation), the arterial and venous carbon dioxide values are not increased above the usual normal levels. This might be expected from the fact shown by Krogh and Krogh (1910) that the lungs maintain approximate equality of carbon dioxide tension between arterial blood and alveolar air much more readily than they maintain equality of oxygen tension.

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EFFECT OF CALCIUM ON THE COMPOSITION OF THE EGGS AND CARCASS OF LAYING HENS.

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In connection with certain studies concerning the growth of the White Leghorn hen, an experiment was planned to determine the effect produced by grit, oyster shell, and limestone on the composition of the eggs laid when fed in connection with an ordinary ration used for laying hens; and also to determine what would be the ultimate effect on the body of the hens of the continued laying of eggs in the absence of calcium other than that contained in the food. In other words, it was proposed to determine the composition of eggs of hens whose supply of calcium was limited to that contained in a dry mash and mixed grains (having a low calcium content) as compared with the composition of the eggs from hens receiving all the calcium they might desire from oyster shell and ground limestone. It also seemed important to determine to what degree the continued laying of eggs, on a limited intake of calcium, would lower the calcium content of the carcass of the hen before she should stop laying. The corresponding distribution of magnesium and phosphorus was also to be determined.

With these ends in view, forty pure bred, White Leghorn pullets were selected from the same incubator hatching and divided into four lots of ten each, having approximately the same development and vigor. These were placed in four separate hen houses which were identical in every way, and during the entire experiment the pullets were not allowed access to the ground, thus eliminating any possible chance of their obtaining inorganic material from undesired sources. These lots were designated Nos. 1, 2, 3, and 4 and all received the same ration; namely, a dry mash composed of 6 parts corn-meal, 3 parts bran, 3 parts middlings,

5 parts meat meal, and 5 parts charcoal; and a grain mixture of 16 parts wheat, 16 parts cracked corn, and 8 parts oats. These foods were supplemented in Pen 2 with grit, in No. 3 with grit and oyster shell, and in No. 4 with grit and limestone; whereas Pen 1 received no additional mineral matter. The percentages of calcium, magnesium, and phosphorus in the foods and the supplemental mineral material are given in Table I, calculated as the oxides.

At the beginning of the experiment, December 1, 1918, separate analyses were made of the shell and of the contents of an average egg. Also a representative pullet was killed and the head, skin, feathers, feet, and intestines with the contents of the gizzard were discarded, to eliminate any extraneous matter. The two large upper bones (femur and tibia) of both legs were dissected

TABLE I.

Analysis of the Materials Fed.

Material.	Crude ash.	Calcium oxide (CaO).	Magnesium oxide (MgO).	Phosphorus pentoxide (P_2O_5).
	per cent	*per cent*	*per cent*	*per cent*
Grain mixture	2.37	0.0013	0.0006	0.0078
Mash "	7.75	0.0192	0.0024	0.0182
Grit		1.00	0.82	0.09
Oyster shell		51.85	0.37	0.12
Limestone		47.95	1.00	0.18

out and, after being freed from adhering material, were analyzed separately from the remaining part of the carcass. During the progress of the experiment, when marked visible changes appeared in the hens, an egg was obtained on the same day from each of the four lots and, after being carefully cleaned with distilled water, the shells and their contents were separately analyzed for calcium, magnesium, and phosphorus. Also, whenever a hen broke down, she was killed and analyzed as stated above.

As the experiment advanced it was noticed that the general condition of Lots 1 and 2 was not so good as that of Lots 3 and 4 which received the calcium supplement. It will be seen in Table II that deaths occurred in each lot except Lot 3; they occurred from various causes which are not of such a character as would result directly from lack of proper nourishment. In Lot 1 there

was no instance of a complete breakdown similar to those that occurred in Lot 2 on March 9th and May 20th. It seems most probable that this breaking down in Lot 2 was due to individual weakness rather than to the grit supplementing their ration, which was the only point in which the ration differed from that of Lot 1. The average number of eggs laid per hen, per month, was approximately the same in Lots 1 and 2, while in Lot 4 the average was 13.5 per cent greater than that of Lot 3, which may or may not be attributed to individual variation.

In all lots, March and April seem to be the periods of greatest average egg production and while this production was less in all

TABLE II.

Mortality and Egg Record.

Lot No.	December.			January.			February.			March.			April.			May.			Total eggs laid in 6 months.	Average No. of eggs per hen.
	No. of hens.	Total eggs.	No. of eggs per hen.	No. of hens.	Total eggs.	No. of eggs per hen.	No. of hens.	Total eggs.	No. of eggs per hen.	No. of hens.	Total eggs.	No. of eggs per hen.	No. of hens.	Total eggs.	No. of eggs per hen.	No. of hens.	Total eggs.	No. of eggs per hen.		
1	10	0	0	10	16	1.6	10	54	5.4	9*	80	8.9	9	120	13.3	9	21	2.3	291	31.5
2	10	2	0.2	9†	27	3.0	8†	51	6.4	7‡	71	10.1	7	41	5.8	7	37	5.3	229	30.6
3	10	0	0	10	20	2.0	10	86	8.6	10	171	17.1	10	122	12.2	10	89	8.9	488	48.8
4	10	3	0.3	10	43	4.3	9*	73	8.1	9	143	15.9	9	137	15.2	8*	102	12.8	491	56.4

* Cause of death unknown.

† Death caused by chicken pox.

‡ This hen was killed after breaking down and was analyzed; see Table V.

lots during the month of May than it was in March or April, yet it can plainly be seen that the lack of mineral matter increased the difference.

As will be seen in Tables III and IV, which give the analyses of the egg shells and their contents, an egg was analyzed December 1, which marked the starting of the experiment, and on February 12, or approximately 10 weeks later, an egg was analyzed from each lot, since it was noticed at this time that the general appearance of Lots 1 and 2 did not equal that of Lots 3 and 4. This difference became more exaggerated as time passed until, on March 8th, a hen in Lot 2 broke down. She was unsteady and remained in a squatting position, unless disturbed. On the day

following, the hen could not stand and, since her appetite was practically gone, she was killed and analyzed as described elsewhere. The results of these analyses are shown in Table V. At this point an egg was obtained from each lot and analyzed.

TABLE III.

Analyses of Shells of Eggs from All Lots.

Date.	Lot No.	Total shell.	Total ash.		Calcium, as CaO, in ash.		Magnesium, as MgO, in ash.		Phosphorus, as P_2O_5, in ash.	
		gm.	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*
Dec. 1, 1918	Normal.	4.702	2.753	58.55	2.720	98.10	0.019	0.71	0.019	0.70
Feb. 12, 1919	1	5.129	2.930	57.13	2.850	97.25	0.018	0.62	0.020	0.69
	2	4.660	2.586	55.47	2.525	97.65	0.016	0.62	0.013	0.51
	3	5.742	3.083	53.69	3.030	97.30	0.023	0.77	0.020	0.65
	4	5.853	3.191	54.53	3.129	98.05	0.019	0.61	0.023	0.74
Mar. 8, "	1	4.685	2.541	54.25	2.475	97.40	0.018	0.74	0.015	0.59
	2	3.910	2.119	54.20	2.093	98.80	0.012	0.61	0.011	0.54
	3	5.725	3.172	55.41	3.123	98.45	0.019	0.63	0.012	0.40
	4	4.563	2.522	55.28	2.482	98.40	0.013	0.54	0.018	0.73
" 22, "	1	3.608	1.934	53.61	1.895	98.00	0.013	0.72	0.012	0.67
	2	3.775	2.035	53.92	1.986	97.95	0.017	0.87	0.011	0.57
	3	5.879	3.222	54.82	3.164	98.20	Lost.	Lost.	0.013	0.41
	4	5.903	3.189	54.03	3.119	97.80	0.024	0.76	0.022	0.70
May 20, "	1	*								
	2	3.050	1.604	52.61	1.577	98.30	0.013	0.81	0.009	0.62
	3	4.664	2.497	56.12	2.447	98.00	0.022	0.89	0.015	0.60
	4	5.269	2.956	56.11	2.885	97.60	0.027	0.93	0.028	0.97
June 1, "	1	2.949	1.564	53.03	1.524	97.50	0.013	0.89	0.009	0.60
	2	2.797	1.542	55.14	1.500	97.20	0.015	1.03	0.013	0.86
	3	3.233	1.954	60.46	1.918	98.15	0.015	0.80	0.017	0.90
	4	4.320	2.519	58.32	2.471	98.10	0.022	0.90	0.016	0.65

* The shell of the egg obtained from Lot 1 on May 20 was so thin that it broke in handling.

On March 22, May 20, and June 1 an egg was obtained from each lot and analyzed and, since, on June 1, the hens in Lots 1 and 2 were in a state bordering on a general breakdown and had practically ceased laying eggs, the experiment was brought to a

close and an average hen from each lot was killed and analyzed. In Table VI will be found the analyses of the carcasses and leg bones of hens from each of the four lots, made 6 months after the start of the experiment.

TABLE IV.

Analyses of the Contents of Eggs from All Lots.

Date.	Lot No.	Total contents.	Total ash.		Calcium, as CaO, in ash.		Magnesium, as MgO, in ash.		Phosphorus, as P_2O_5, in ash.	
		gm.	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*
Dec. 1, 1918	Normal.	43.216	0.410	0.95	0.033	8.12	0.006	1.46	0.164	40.05
Feb. 12, 1919	1	44.095	0.423	0.96	0.034	8.05	0.005	1.38	0.157	37.20
	2	41.711	0.354	0.85	0.030	8.31	0.005	1.58	0.155	44.00
	3	47.022	0.456	0.97	0.038	8.50	0.007	1.57	0.181	39.77
	4	39.612	0.380	0.96	0.032	8.55	0.005	1.33	0.161	42.40
Mar. 8, "	1	49.702	0.412	0.83	0.030	7.44	0.006	1.45	0.164	39.71
	2	52.110	0.437	0.84	0.035	8.04	0.004	1.78	0.144	33.00
	3	44.201	0.419	0.95	0.033	7.93	0.004	1.13	0.184	44.04
	4	42.846	0.372	0.87	0.028	7.51	0.005	1.34	0.164	44.11
" 22, "	1	37.800	0.378	1.00	0.030	7.98	0.004	1.12	0.141	37.51
	2	44.315	0.398	0.90	0.025	6.45	0.003	0.81	0.148	37.31
	3	52.080	0.479	0.92	0.039	8.34	0.005	1.22	0.194	40.50
	4	52.413	0.487	0.93	0.038	8.05	0.004	0.87	Lost.	Lost.
May 20, "	1	*								
	2	39.788	0.338	0.85	0.023	6.86	0.005	1.52	0.125	37.20
	3	45.042	0.427	0.95	0.035	8.32	0.005	1.37	0.206	48.20
	4	44.215	0.415	0.97	0.034	8.31	0.004	1.12	0.206	49.60
June 1, "	1	37.161	0.308	0.83	0.020	6.50	0.003	1.14	0.121	39.50
	2	35.083	0.287	0.82	0.021	8.10	0.003	1.06	0.113	46.00
	3	37.521	0.352	0.94	0.037	10.80	0.004	1.40	0.162	46.10
	4	51.019	0.474	0.93	0.033	7.19	0.005	1.09	0.233	49.20

* The shell of the egg obtained from Lot 1 on May 20th was so thin that it broke in handling.

In analyzing the separate portions of the ash of the materials under consideration, the phosphorus was determined by the method of the Association of Official Agricultural Chemists,[1]

[1] Wiley, H. W., Official and provisional methods of analysis, *U. S. Dept. Agric., Bureau of Chemistry, Bull. 107*, revised, 1912.

TABLE V.

Analyses of Normal Hen and a Hen That Had Broken Down in Lot 2.

Date.	Lot No.	Part analyzed.	Total weight of part analyzed.	Total ash.		Calcium, as CaO, in ash.		Magnesium, as MgO, in ash.		Phosphorus, as P_2O_5, in ash.	
			gm.	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*
Dec. 1, 1918	Normal.	Carcass.	727.00	20.305	2.79	7.563	37.25	0.213	1.05	7.750	38.17
	"	4 leg bones.	24.34	7.235	29.70	3.766	52.05	0.048	0.67	2.841	39.27
Total			751.34	27.540		11.329		0.261		10.591	
Mar. 9, 1919	Broken down in Lot 2.	Carcass.	513.50	14.934	2.91	5.354	35.85	0.158	1.06	6.318	42.31
		4 leg bones.	19.05	3.783	19.86	1.920	50.75	0.024	0.64	1.494	39.50
Total			532.55	18.717		7.274		0.182		7.812	

TABLE VI.

Analyses of Carcasses and Leg Bones of Hens from All Lots, at the End of the Experiment, June 1, 1919.

Date.	Lot No.	Part analyzed.	Total weight of part analyzed.	Total ash.		Calcium, as CaO, in ash.		Magnesium, as MgO, in ash.		Phosphorus, as P_2O_5, in ash.	
			gm.	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*
June 1, 1919	1	Carcass.	629.00	23.269	3.69	7.213	31.00	0.235	1.01	7.574	32.55
		4 leg bones.	32.00	8.586	26.83	4.602	53.60	0.059	0.69	3.484	40.58
	Total		661.00	31.855		11.815		0.294		11.058	
	2	Carcass.	552.00	15.274	2.76	4.643	30.4	0.139	0.91	4.803	31.45
		4 leg bones.	24.90	4.509	18.10	2.371	52.6	0.025	0.57	1.739	38.58
	Total		576.90	19.783		7.014		0.164		6.543	
	3	Carcass.	836.00	27.020	3.23	10.470	38.75	0.375	1.38	10.851	40.16
		4 leg bones.	33.00	11.338	34.36	6.240	55.04	0.329	2.91	4.382	38.66
	Total		869.00	38.358		16.710		0.704		15.233	
	4	Carcass.	731.00	26.400	3.61	9.570	36.25	0.398	1.51	9.240	35.00
		4 leg bones.	32.00	8.005	25.02	4.419	55.20	0.249	3.11	3.216	40.18
	Total		763.00	34.405		13.989		0.647		12.456	

while calcium and magnesium were determined according to the method of McCrudden.[2]

From Tables III and IV it will be seen that there is little variation in the percentages of ash, calcium, magnesium, and phosphorus in the egg shells and the contents of the eggs in all four lots during the 6 months over which the experiment lasted. However, there was a decided tendency in Lots 1 and 2 for the total weights of the shell to become smaller as the experiment advanced; in other words, the shell became progressively thinner. On May 20 the shell of the egg obtained from Lot 1 was so thin that it broke in handling and was lost. In the case of Lots 1 and 2, on June 1, the eggs were handled in cotton-padded boxes, to prevent breaking. In no case was a shell-less egg obtained from these four lots.

In Table V we see that the total weight of the parts of the hen which broke down on March 9 was 218.79 gm. less than the normal hen that was over 3 months younger. One of the most interesting points in the comparison of the analyses of these two hens is the great difference in the percentage of ash, while there is practically no difference in the percentage of calcium, magnesium, and phosphorus in the ash. In other words, there seems to be a stable equilibrium in this connection which exists even though there is a marked difference in the total quantity. As will be seen in Table VI, magnesium fails to adhere to this rule.

In Table VI the comparative analyses of the carcasses and leg bones of hens from Lots 1, 2, 3, and 4 show that the hens of Lots 1 and 2 weigh appreciably less than those of Lots 3 and 4 and that there is a general depletion of the quantities of ash, calcium, magnesium, and phosphorus in Lots 1 and 2.

It would seem from a study of the foregoing figures that the following conclusions are justified.

1. Laying hens whose supply of calcium is limited to that naturally occurring in the food will continue laying eggs until there is a general depletion of magnesium, phosphorus, and calcium in their bones and carcasses.

[2] McCrudden, F. H., The quantitative separation of calcium and magnesium in the presence of phosphates and small amounts of iron devised especially for the analysis of foods, urine and feces, *J. Biol. Chem.*, 1909–10, vii, 83.

2. As long as the economy of the hens permitted the formation of an egg shell, the contents of the egg remained reasonably constant, thereby permitting an average supply of calcium, magnesium, and phosphorus for the proper development of the embryo of the chick.

3. 10 weeks elapse before there is a noticeable change in the general condition of hens receiving no mineral matter other than that naturally occurring in the food such as that fed to Lots 1 and 2.

4. It would seem most probable that the breaking down of certain hens before the expiration of 6 months was caused by individual weakness.

5. Since no shell-less eggs were laid in Lots 1 and 2, it would indicate that the lack of calcium is not the fundamental cause of their formation.

6. The percentages of calcium and of phosphorus in the bones of the hens in all lots were reasonably constant during this experiment, thus indicating a stable equilibrium between the two elements.

7. The continued laying of eggs, under the calcium restrictions of this experiment, does not materially alter the percentage mineral composition of the egg shells or their contents. There is, however, a gradual thinning of the egg shells.

8. Under the conditions governing this experiment, the addition of limestone or oyster shell to the ration increases the production of eggs 69.4 per cent as shown by the average egg production of Lots 1 and 2 when compared with that of Lots 3 and 4.

NOTE ON A SHORT MODIFICATION OF THE OFFICIAL CHLORINE METHOD FOR FEEDS, FECES, AND URINE.

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The determination of chlorine in blood or body fluids has resulted in a modification of the Volhard method by Rappleye[1] and in the development of an iodometric method by McLean and Van Slyke[2] both of which aim to give better and more distinct end-points than the above official method[3] and therefore more accurate results.

The concentration of the NaCl estimated by them in blood involved a magnitude of 400 to 600 mg. per 100 cc. of liquid. The magnitude of chlorine estimated in feeds, feces, and swine urine is less than 100 mg., consisting respectively of 39, 19, and 104 mg. or the actual amount estimated per determination was 3.8, 3.6, and 14.0 mg. The estimation of such small quantities by the official Volhard method as usually done does not give as consistent results as desired.[4]

This is chiefly because of the volumetric strength of the reagents employed, the indefiniteness of the end-point in titration, and the difficulty of washing out excess $AgNO_3$ from the precipitated AgCl without obtaining a slightly turbid filtrate which renders the end-point less distinct. The process of washing the excess $AgNO_3$ out is also long and tedious resulting in a bulky filtrate which gives a less distinct end-point.

[1] Rappleye, W. C., *J. Biol. Chem.*, 1918, xxxv, 509.

[2] McLean, F. C., and Van Slyke, D. D., *J. Biol. Chem.*, 1915, xxi, 361.

[3] *J. Assn. Offic. Agric. Chem.*, 1915–16, i, p. xxxii.

[4] Halverson, J. O., and Schulz, J. A., *J. Am. Chem. Soc.*, 1919, xli, 440.

For these reasons the modification given below was adopted. This consisted in eliminating the step of washing excess $AgNO_3$ out from the AgCl precipitate and substituting titration of an aliquot of the clear filtrate directly after filtering off the AgCl precipitate. This procedure gives the same results as the official method when the latter is refined by using weaker reagents of $AgNO_3$ and of NH_4CNS, and using a burette with the latter reading to 0.05 cc. in which the NH_4CNS is capable of being estimated to 0.01 cc. Further, a definite smaller volume of solution is always titrated.

The Proposed Modification.

After the digestion of the alkaline ash with dilute nitric acid the solution is filtered into a beaker and the residue washed with hot water to a total volume not exceeding 75 cc. To the boiling solution on the hot plate is gradually added 0.05 N $AgNO_3$, chlorine being slowly precipitated. The volume is then reduced to 25 or 30 cc. by boiling which allows the precipitate to coagulate and gives a perfectly *clear* solution. The solution is allowed to cool, when it is carefully transferred to a 100 cc. volumetric flask, made up to mark, and is then gently mixed by inverting the flask several times. Let stand 3 to 4 hours or, if convenient, over night thus allowing the precipitate to settle. Filter through a dry filter paper into a dry beaker. Pipette out 95 cc. into a 200 cc. Erlenmeyer flask.[5] Add 2 cc. of ferric nitrate indicator,[6] titrate the excess $AgNO_3$ with NH_4CNS, which is one-half the strength of the $AgNO_3$,[7] against a white background using a burette reading to 0.05 cc. and capable of being estimated to 0.01 cc.

The number of cc. of NH_4CNS used, increased by $\frac{100}{95}$, gives the equivalent cc. for the total charge taken. Cc. of $AgNO_3$ − (cc. of $NH_4CNS \times \frac{100}{95} \times$ factor) = cc. of $AgNO_3$ used.

[5] A 47.5 cc. pipette is conveniently used here.

[6] 125 cc. of concentrated nitric acid are added to 325 cc. of water; boiled until colorless. 50 cc. of a saturated solution of ferric nitrate are added.

[7] 0.05 N $AgNO_3$ is used; 1 cc. is approximately equivalent to 1.5 mg. of chlorine.

In Table I are given results by the refined official Volhard method and also with the proposed modification included. It is seen that the small differences, part of a milligram, per 100 gm. or per 100 cc. are of the same order of magnitude as obtained by McLean and Van Slyke.[2]

TABLE I.

*Comparison of the Modified Chlorine Method with the Official Chlorine (Volhard) Method.**

Material tested.	Quantity taken.	Chlorine by official method.†		Quantity taken.	Chlorine by modified method.		Difference per 100 gm. or cc.
		Found.	Per 100 gm. or cc.		Found.	Per 100 gm. or cc.	
		mg.	*mg.*		*mg.*	*mg.*	*mg.*
Corn	10.00 gm.	3.99	39.9	10.00 gm.	4.08	40.8	0.9
Linseed oil meal	10.00 "	3.82	38.2	10.00 "	3.89	38.9	0.7
Wheat middlings	10.00 "	3.61	36.1	10.00 "	3.69	36.9	0 8
Swine feces 1	18.00 "	3.84	21.3	16.97 "	3.65	21.5	0.2
" " 2	18.09 "	3.24	17.9	18.31 "	3.47	18.9	1.0
" " 3	17.95 "	3.06	17.0	17.64 "	3.09	17.5	0.5
" " 4	18.04 "	3.74	20.7	18.61 "	3.96	21.3	0.4
" urine 5	10.00 cc.	9.49	94.9	10.00 cc.	9.43	94.3	0.6
" " 6	10.00 "	9.13	91.3	10.00 "	9.22	92.2	0.9
" " 7	10.00 "	12.10	121.0	10.00 "	12.18	121.8	0.8
" " 8	10.00 "	10.70	107.0	10.00 "	10.74	107.4	0.4

* All samples were done in triplicate. Averages are given.

† 1 cc. of $AgNO_3$ was equivalent to 1.492 mg. of chlorine while the ratio for NH_4CNS was 1.2755; the ratio of NH_4CNS for the modified method was 0.5076.

Both the official Volhard and the modified method were checked against salts of known chlorine content, also against dried C. P. NaCl. The proposed method varied from a few hundredths to 0.6 or 0.7 per cent while the official Volhard method by the usual procedure varied 1 to 2 per cent from theory.

By this method clear solutions of small definite volumes for titration are obtained thus avoiding the frequent turbidity encountered and the tedious washing of the AgCl precipitate free from the excess $AgNO_3$.

The authors wish to thank Dr. E. B. Forbes for courtesies rendered.

A COMPARATIVE STUDY OF HEMOGLOBIN DETERMINATION BY VARIOUS METHODS.

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The regeneration of hemoglobin and red cells following simple anemia has been studied in this laboratory for more than 2 years. A preliminary report of this work by Whipple and Hooper (13) has appeared and it is obvious that this curve of hemoglobin regeneration can be influenced by a number of diet factors. In this work it is essential that there be an accurate determination of hemoglobin. For this reason a comparative study of many hemoglobin methods was undertaken. It is believed that the method finally adopted for this work will be of value to other workers in the experimental field as well as to hospital and school laboratories where routine hemoglobin readings are so frequent. It need not be stated that much of the work expended upon routine hemoglobin determination is a total loss because the instrument used has not been standardized or the method is inaccurate. Too little is known as to the normal hemoglobin value in human beings as affected by age, altitude, climate, etc. No comparison is possible until some accurate standard is adopted for the routine work.

The earlier anemia work in this laboratory was done with Sahli's method using his modification of Gowers' instrument. It was soon apparent that incorrect results were being obtained and that the hemoglobin percentages were considerably higher than they should be when correlated with the other blood findings. The standard color tubes when checked against the oxygen capacity method of Van Slyke (12) showed great variations in color density. New tubes were purchased and when standardized showed much fading varying from 5 to 20 per cent. The

results obtained with these tubes were therefore sufficiently erroneous to warrant the discarding of this method for hemoglobin determination.

The Palmer carbon monoxide method was next tested and accurate results were obtained, provided the standard solutions were frequently checked.

Since the publication of Newcomer's method based on spectrophotometric data this method has also been carefully investigated, as well as a combination of Palmer's and Sahli's method with slight modifications described below.

History of Methods.

I. Acid Hematin Method.—Sahli (10), finding that methods employing artificial color standards were not satisfactory, brought forth the acid hematin method. Hemoglobin is converted into acid hematin by the addition of 0.1 N HCl and then compared with a standard of like material. Numerous criticisms of the method have appeared and as many modifications been offered. Berczeller (2) claimed that the presence of lipoids alters readings. Stäubli (11) called attention to the time factor for maximum color development. Palmer (9) claimed that the standard is not permanent, that there is considerable delay in maximum color development, and that the method is not applicable for blood of different species. Haessler and Newcomer (4) offered a modification in the instrument used, using eleven standard tubes of different concentrations for comparison. Lilliendahl-Petersen (7) employed Sahli's principle in a Tallquist form. Newcomer (8) recently published a method of hemoglobin determination by comparing an acid hematin suspension of blood with a piece of brown-colored glass of definite thickness. The method is based on spectrophotometric data. The comparison is made with a Duboscq type of colorimeter.

II. Carbon Monoxide Method.—Hoppe-Seyler in 1892 (6) published his procedure of accurately determining hemoglobin in the form of carbon monoxide hemoglobin. The technical difficulties involved were too numerous for general adoption of the method.

Haldane (5) 8 years later revived Hoppe-Seyler's principle of hemoglobin determination but in a much simpler form. He used Gowers' instrument.

Palmer (9) in 1918 published a method which has found much favor. The principle is that of Hoppe-Seyler's procedure; *i.e.*, a color comparison of carbon monoxide hemoglobin solution with a standard of known hemoglobin content. Ammonia solution is used as a diluent instead of water. The color comparison is made in a Duboscq colorimeter.

EXPERIMENTAL.

I. Palmer Method.—We have used Palmer's method with only slight modifications. All experiments carried on during this investigation have been done on dogs. All blood is obtained by venous puncture. About 10 cc. of blood are drawn from the jugular vein with a glass syringe and emptied into a graduated centrifuge tube containing 2 cc. of a 1.6 per cent sodium oxalate solution. The plasma obtained by centrifugalization is carefully pipetted off, the tube slightly tilted, and a 1 cc. calibrated pipette of small lumen quickly inserted with the finger closing the opening at the upper end. Blood is slowly drawn up to the 1 cc. mark, the pipette is thoroughly wiped on the outside, and its contents are transferred into a small test-tube. The pipette is carefully rinsed in 2 cc. of N salt solution previously measured with this same pipette and emptied into a test-tube similar to the one containing the blood. This salt solution, now containing some red cells, is then carefully added to the 1 cc. of blood and the whole thoroughly mixed avoiding of course too vigorous shaking. Extreme care must be taken with this procedure so that the suspension of packed red blood cells is truly a dilution of one in three. The latter was ascertained to be the most convenient dilution, for the hemoglobin of our normal dogs is usually considerably over 100 per cent, frequently showing readings of 130 to 140 per cent. From the diluted blood cells suspension a 1 per cent solution of blood is made. 1 cc. of the diluted blood, 1:3, is drawn up into the same pipette used for diluting the packed cells as well as for measuring the original amount, is transferred to a 100 cc. volumetric flask containing

the 0.4 per cent ammonia solution, and made up to the 100 cc. mark with this same diluent. The solution is thoroughly mixed and at once saturated with carbon monoxide and read immediately. The percentage of hemoglobin obtained is multiplied by three—the packed red cells having been previously diluted 1:3—and this figure again multiplied by the red cell percentage of the blood computed from the hematocrit readings. In order to determine the accuracy of this apparently roundabout procedure, which is employed to prevent further bleeding for hemoglobin determination, readings were made from whole blood collected from the vein directly into a vessel containing sufficient dry sodium oxalate to prevent clotting and compared with the figures

TABLE I.*

Hemoglobin of packed red blood cells diluted 1:3.	Undiluted packed red cells.	Red cells from hematocrit.	Estimated hemoglobin from packed cells.	Hemoglobin of whole blood direct.	Difference.
per cent	*per cent*	*per cent*	*per cent*	*per cent*	
69	207	46.0	95	94	+1
57	171	48.0	82	83	−1
67	201	40.0	80	80	0
59	177	47.2	84	84	0
62.1	186.3	50.5	94	94	0
76.9	230.7	42.6	98	97	+1
63.0	189	38.4	73	73	0

* This work has been repeated and similar results have been obtained.

obtained by our means. Table I demonstrates the accuracy of the method as we employ it. It is obvious that by increasing the amount of hemoglobin used we tend to diminish any error of the method. 1 cc. of packed red cells should give more accurate readings than 20 mm. of whole blood.

Palmer's technique for the preparation of his standards has been closely adhered to. Palmer used either defibrinated ox or human blood. We have tried whole as well as defibrinated blood. Furthermore, blood obtained from different species has been investigated. In some cases blood has been obtained aseptically and the standard prepared from it with all aseptic precautions. With other standards only the usual cleanliness has been exercised.

The figures in Table II demonstrate the amount of fading of standards during the time of observation.

TABLE II.

Change in Color Value of Palmer Standards of Hemoglobin.

Standard No.	Source of blood.	Aug. 8, 1918.	Aug. 23, 1918.	Sept. 20, 1918.	Oct. 21, 1918.	Nov. 15, 1918.	Dec. 24, 1918.	Jan. 24, 1919.	Mar. 5, 1919.	April 1, 1919.	May 16, 1919.	June 23, 1919.	Aug. 5, 1919.	Sept. 7, 1919.	Oct. 21, 1919.	Total fading.	Remarks.
																per cent	
I	Dog (whole).	100	99	90	75	73	74	74	72	71						29	Discarded. Slight brownish tinge last 3 months.
II	Goat " sterile.		100	85	80	73	74	74	75	75	76	75	75	75		26	Discarded. Slight brownish tinge last 3 months.
III	Dog (defibrinated).			100	99	89	87	86	86	80	80	81	82	82	82	20	No color change.
IV	Human "					100	93	89	89	84	84	84	85	85	84	16	" " "
V	Sheep "							100	98	91	91	92	90	90	90	10	" " "
VI	Dog (whole).								100	93	88	87	85	86		15	Spoiled.
VII	" (defibrinated).									100	100	100	100	100	100	0	No color change.
VIII	" "										100	99	99	96	95	5	" " "

Discussion of Table II.

No. I showed the greatest fading, 29 per cent in 8 months, the maximum change taking place during the first 2 months. The slight color change did not interfere with color comparison.

No. II was prepared from goat blood with aseptic precautions and kept sterile throughout the experiment. Neither the fragility of red cells nor bacterial decomposition seems to play an important rôle in the fading of color. The change was nearly as much as with No. I, the maximum fading taking place during the first 3 months.

The curve of No. III is more promising, defibrinated blood showing slightly better keeping qualities than whole blood. The fading was not so rapid nor so much as in Standards I and II, nor was any color change apparent.

No. IV prepared from pooled defibrinated human blood showed about the same stability of color as defibrinated dog blood. The greatest change took place during the first 2 months; after that the fading was slight.

We were more successful in keeping No. V, prepared from defibrinated sheep blood, for the maximum fading was but 10 per cent as compared with 16 and 20 per cent when using defibrinated human and dog blood.

No. VI, having as its source whole dog blood, faded 15 per cent during 6 months, not so much as Standards I and II also prepared from whole blood, but still slightly more than when the blood was previously defibrinated.

Standard VII again prepared from defibrinated dog blood was the only one which remained unchanged during the period of observation, 7 months.

Standard VIII, for which defibrinated dog blood was again employed, showed a fading of 5 per cent during 5 months. While more promising than the earlier ones, still the change is too much for accurate hemoglobin determinations.

Another standard prepared from whole dog blood to determine again the difference obtained with whole and defibrinated blood is but 2 months old. The fading already amounts to 7 per cent during this period.

II. Acid Hematin Method.—Because of the uncertain stability of carbon monoxide hemoglobin solutions we have attempted to solve the problem by searching for a more stable color standard. In view of Sahli's work acid hematin was tried again. It offers an easier color comparison than do the reds of oxyhemoglobin and carbon monoxide hemoglobin. Sahli in his original work obtained satisfactory results, as did several of his coworkers. We have combined Palmer's method with Sahli's principle, that is, determined hemoglobin in the form of acid hematin, employing Palmer's method of standardization. The sealed tubes containing dilute acid hematin suspension as purchased (Sahli instrument) are unsatisfactory as was pointed out in the history of the methods. The thought that acid hematin in a more concentrated form might not fade so readily arose, and therefore 5, 10, and 20 per cent suspensions were investigated. Our method of procedure was as follows:

The oxygen capacity of a sample of dog's blood was determined by Van Slyke's method and the hemoglobin content computed therefrom. An acid hematin standard in the form of a 20 per cent suspension, so diluted that a 1 per cent dilution prepared from it would read 100 per cent, was made. Because of our experience with whole and defibrinated blood in the preparation of standards for the carbon monoxide method we employed defibrinated blood for our first standard. It is well known and has been mentioned in the history of acid hematin methods that the time factor for allowing the maximum color of acid hematin to develop plays a very important rôle. This standard, after preparation and dilution, was allowed to stand 24 hours to insure correct readings. A 1 per cent dilution prepared from the 20 per cent suspension was of course employed for direct color comparison. The same time factor was used for the blood to be tested. A 1 per cent suspension of blood is used for the determination of hemoglobin. 0.1 N HCl is employed throughout the procedure, for the original standard suspensions as well as for all further dilutions. The strength of HCl used within a certain limit is immaterial. We tried N, 0.1 N, and 0.5 N and obtained identical readings. Stronger solutions than N caused precipitation. The entire amount of diluent is used at once, that is, 1 cc. of diluted packed red cells is discharged

into the volumetric flask containing about 50 cc. of 0.1 N HCl, then mixed, and the volume made up to the 100 cc. mark with 0.1 N HCl. The suspension is then allowed to stand 24 hours in the ice chest, thoroughly shaken again, for it must be remem-

TABLE III.

Hemoglobin.

Palmer's method.	Author's modification.
per cent	*per cent*
75	74
70	70
77	76
77	77
74	73
73	73
74	74
75	74
76	75
73	73
64	64
63	63
76	76
45	45
60	60
84	85
69	69

TABLE IV.

Van Slyke's oxygen capacity method.	Palmer's method.	Author's modification.
per cent	*per cent*	*per cent*
113	113	114
117	118	117
99	98	99
124	124	124
109	108	109

bered that we are dealing with a suspension and not a solution, and then read. The method of hemoglobin determination is exactly like that of Palmer's method and the readings obtained are almost identical as illustrated by the figures given in Tables III and IV.

The source of light used for color comparison makes little difference. Light from a northern exposure or that originating from a nitrogen-filled bulb filtered through "Daylite" glass gives equally good readings. The slight turbidity of the acid hematin suspension, and it is very slight in a 1 per cent dilution, does not in the least interfere with an accurate color comparison.

Different standards have been prepared, 5, 10, and 20 per cent suspensions, defibrinated as well as whole blood, also blood from different species. In fact, standards have been made up from the same sample of blood, one diluted for carbon monoxide hemoglobin determinations and the other in the form of acid hematin suspensions. The standards have been checked up once a month as those for Palmer's method. Material for the 1 per cent dilution has been withdrawn from the stock bottles once each week, and the container resealed with paraffin. On prolonged standing some of the hematin settles to the bottom of the container but when the mixture is thoroughly shaken again the readings obtained are unchanged. It is of course very essential that all acid hematin suspensions, whether dilute or concentrated, are thoroughly mixed before using.

The keeping qualities, or rather stability of color density, are best demonstrated by Tables V, VI, VII, and VIII.

Standard IVa (Table V) faded 4 per cent during a time interval of 11 months. The change was apparent during the 1st month. In the same period of time the carbon monoxide standard had faded 16 per cent.

These tables (V, VI, VII, and VIII) demonstrate the keeping qualities of our acid hematin mixtures. Up to the present time the last three have remained practically unchanged. We shall continue to check up these suspensions once each month in order to determine just how long they will remain stable.

A 1 per cent standard diluted from Standard VIIa at the time of preparation, April 1, 1919, and simply kept in an Erlenmeyer flask in the ice chest still read 100 per cent on October 21, 1919. Another 1 per cent suspension diluted from the defibrinated sheep blood standard and kept under the same conditions as the one mentioned above also remained unchanged during the period of observation, 4 months. For exactly how long a period the 1 per cent dilution would remain stable we do not at present

know. We consider it safe to make up our 1 per cent suspensions from the stock mixture once each month. It may be of some importance that all these standards were preserved in an

TABLE V.

Standard IVa; Acid Hematin. Defibrinated Human Blood Prepared Nov. 25, 1918.

Date.	New standard prepared.	Standard IVa.	Amount of fading.	Amount of fading of carbon monoxide standard.	Remarks.
1918			*per cent*	*per cent*	
Dec. 24	100	97	3	7	Carbon monoxide standard was prepared from the same sample of blood.
1919					
Jan. 24	100	97	3	11	
Mar. 5	100	96	4	11	
Apr. 1	100	97	3	16	
May 15	100	96	4	16	
June 23	100	97	3	16	
Aug. 6	100	96	4	15	
Sept. 7	100	96	4	15	
Oct. 21	100	96	4	16	

TABLE VI.

Standard Va; Acid Hematin. Defibrinated Sheep Blood Prepared Jan. 24, 1919.

Date.	New standard prepared.	Standard Va.	Amount of fading.	Amount of fading of carbon monoxide standard.	Remarks.
1919			*per cent*	*per cent*	
Mar. 5	100	100	0	2	Carbon monoxide standard was prepared from the same sample of blood.
Apr. 1	100	100	0	9	
May 15	100	100	0	9	
June 23	100	100	0	8	
Aug. 6	100	100	0	10	
Sept. 7	100	100	0	10	
Oct. 21	100	100	0	10	

ice chest with fairly constant temperature, fluctuations rarely exceeding 1–4°C.

TABLE VII.

Standard VIa; Acid Hematin. Whole Dog's Blood Prepared Mar. 5, 1919.

Date.	New standard prepared.	Standard VIa.	Amount of fading.	Amount of fading of carbon monoxide standard.	Remarks.
1919			*per cent*	*per cent*	
Apr. 1	100	100	0	7	Carbon monoxide standard prepared from the same sample of blood.
May 15	100	100	0	12	
June 23	100	99	1	13	
Aug. 6	100	100	0	15	
Sept. 7	100	100	0	14	
Oct. 21	100	100	0	Spoiled.	

TABLE VIII.

Standard VIIa; Acid Hematin. Defibrinated Dog's Blood Prepared Apr. 1, 1919.

Date.	New standard prepared.	Standard VIIa.	Amount of fading.	Amount of fading of carbon monoxide standard.	Remarks.
1919			*per cent*	*per cent*	
May 15	100	100	0	0	Carbon monoxide standard prepared from the same sample of blood.
June 23	100	100	0	0	
Aug. 6	100	100	0	0	
Sept. 7	100	100	0	0	
Oct. 21	100	100	0	0	

III. Newcomer Method.—While this investigation was being carried on Newcomer published his new method of estimation of hemoglobin, details of which have been described above. A piece of this brown semaphore glass 0.96 mm. in thickness was tested and gave most satisfactory readings when compared with Palmer's figures as shown in Table IX.

A Dubosecq colorimeter is used in this laboratory and the brown glass inserted above the plunger. The corresponding cup is partially filled with distilled water for the reasons mentioned by Newcomer. As a source of light for these hemoglobin determinations either the lamp containing "Daylite" glass or light from a northern exposure gave equally satisfactory results. While the colors of the glass 0.96 mm. in thickness and the acid hematin suspension of the blood to be tested matched satisfac-

torily, the color is very light, almost a lemon-yellow when matched. It is therefore quite evident that this is somewhat of a disadvantage for darker shades are more easily matched and of course there is no means of regulating the depth of standard color as one does when using liquid mixtures. For example, with our liquid standards set at 10 the resulting readings of the test fluids range usually between 10 and 13, while with the colored glass, at least with the piece of this particular thickness, the readings are around 5 to 7 and are not so accurate as those around 10 or 12 with the Duboscq instrument.

TABLE IX.

Palmer's method.	Newcomer's method (glass 0.96 mm. thick).	Difference.
per cent	*per cent*	
113	114	+1
116	114	−2
99	100	+1
107	108	+1
98	101	+3
117	118	+1
100	100	0
100	100	0
100	101	+1
100	103	+3
100	99.2	−0.8
Average difference		+0.73

The added advantage of course is that all artificial standards like this glass are supposedly permanent in color and this obviates the necessity of liquid standard preparations. Because of the pale color of the standard glass we purchased another piece somewhat thicker—1.02 mm.—hoping to obtain easier readings. While the color was slightly darker in this new piece our readings were not so accurate as is evident from the figures cited (Table X).

The difference in readings (Table X) was more than with the piece 0.96 mm. in thickness, an average of 0.73 higher with the former glass as compared to 2.4 points lower with the thicker one. We feel certain that this larger difference is due to the

fact that with the piece 1.02 mm. thick the color, although darker, is not so readily matched. The suspension of acid hematin demonstrates of course a very slight turbidity, the lack of which is very noticeable when using the heavier glass. The color of the latter is a clear yellowish brown while the acid hematin because of its slight opaqueness seems a somewhat different shade of brown. Some of the readings cited were made by different workers in the laboratory and the same difficulty was voiced by all that the colors do not seem to be quite the same. Two pieces of glass placed one on top of the other, each 1.02 mm. in thickness, increased the difference in readings considerably, as

TABLE X.

Palmer's method.	Newcomer's method (glass 1.02 mm. thick).	Difference.
per cent	*per cent*	
64	62	−2
63	60	−3
76	72	−4
45	47	+2
60	56	−4
84	81	−3
69	66	−3
Average difference		−2.4

well as the difficulty of exact color match. Newcomer (8) states that it is impossible to secure an artificial color match which runs true through a range of thicknesses. While the actual difference in thickness of the pieces of glass purchased—using of course only one at a time—seems slight, a decided difference in color match is apparent.

In view of the above mentioned difficulties we prefer using the liquid acid hematin standards.

DISCUSSION.

In summarizing our observations with the Palmer method it is evident that as long as the standard solutions are prepared once a month very accurate results may be obtained. The method itself is certainly simple and easily carried out by even

comparatively inexperienced laboratory workers. The main disadvantage lies in the color fading of the standard solutions. Among nine different standards observed for a period of from 2 to 13 months we have found but one solution which for nearly 7 months remained unchanged. The remaining eight all faded sufficiently to prohibit their use for accurate work, with one exception possibly—Standard VIII—which demonstrated a fading of only 5 per cent during a 5 months period. Considering the figures presented it seems that better results were obtainable with blood previously defibrinated than with whole blood. For a time interval of 3 months the standard solutions prepared from defibrinated blood demonstrated a fading of from 0 to 13 per cent as compared with 13 to 27 per cent evident in those mixtures originating from whole blood. We have never encountered any difficulty with reference to a true change of color, at least during a period of 6 months. During the last few months of observation a very slight brownish tinge was noticeable but never sufficient to interfere seriously with a color comparison, excepting perhaps Nos. I and VI, which were discarded after 8 months observation. The greatest change in color density in standards prepared from whole blood apparently takes place during the first 2 months and reaches its maximum during the 3rd month. From then on the change is but comparatively little and remains so, in some instances for a year or over. With the defibrinated blood, I think we have a standard of better keeping qualities; the change is not quite so pronounced. Although two of the defibrinated blood mixtures, Nos. III and IV, showed a fading of 11 per cent each during a time interval of 2 months, we have three others where the change was considerably less, one solution demonstrating but a 5 per cent loss of color within 5 months, another remaining unchanged for nearly 7 months, and a third fading 10 per cent in 9 months.

The type of hemoglobin evidently plays no important rôle, dog's blood apparently giving as satisfactory results as goat's, sheep's, or human blood; or rather the other species mentioned offer no more stable hemoglobin solutions than dog's blood.

Bacterial decomposition does not seem to be a very important factor, for blood obtained aseptically and the hemoglobin solution prepared with sterile precautions demonstrated no more

stable qualities than did those standards prepared with only the usual care and cleanliness.

It should be mentioned here that all these stated observations pertain to the stock solutions, the 20 per cent dilutions. We have never attempted to keep the 1 per cent dilutions prepared from the concentrated mixtures for more than a week, at least under experimental conditions existing here. A 1 per cent solution prepared will not fade within a week if kept on ice and resaturated with carbon monoxide each time the container is opened. After that time interval a change does take place which although not apparent to the eye is readily demonstrable when checked against the oxygen capacity method. Appleton (1) states that the 1 per cent solutions prepared by her began to deteriorate in from 2 to 4 weeks. During her investigation the diluted solution was kept saturated by a continual flow of gas. Why such a difference in stability occurs we do not know. Frequent opening of containers and resaturation with CO seem to give no better results than resaturation once a month. While a standard is being used for routine work it is necessary to open the container once each week in order to procure the necessary material for one 1 per cent dilution.

From the tables of acid hematin standards, it is readily seen that, while these acid hematin mixtures may change in time, they certainly have proved themselves to be much more stable than the carbon monoxide solutions. One may with perfect safety and with complete assurance of obtaining accurate results employ these acid hematin suspensions for 6 months at least. The stock mixtures above mentioned will of course be observed to determine just how long they actually remain unchanged.

The method of preparing blood for hemoglobin determinations in the form of acid hematin is slightly simpler than the carbon monoxide method, for the former makes unnecessary the extra step of saturation with carbon monoxide. The slight disadvantage is the time interval necessary for the maximum color of acid hematin to develop. In using large quantities of blood (at least much greater amounts than are used clinically) we have allowed 24 hours. This, however, is not necessary as a 1 hour interval gives accurate reading. We have observed no difference between figures obtained after 60 minutes standing and 24 hours.

The latter time happened to be more convenient in our experimental work. Newcomer published a table with his method showing the exact percentages of color development of acid hematin in given periods of time. He considers 40 minutes as safe.

Palmer in his publication states that blood of different species cannot be used for hemoglobin determinations in the form of acid hematin. As will be seen from our tables, we have standards prepared from human, dog's, and sheep's blood and have compared human blood with both dog's and sheep's blood standards without encountering any difficulty whatsoever. The comparisons have been made in all combinations possible with our material and readings have checked accurately. The figures presented in the tables readily answer the question of stability. While the latter may not be permanent or remain unchanged indefinitely, still it is much less time-consuming to prepare a fresh standard once every 6 months instead of once each month as we have had to do when employing the carbon monoxide method.

The Newcomer method would of course be the best solution of the entire problem, but as mentioned before the color match is not exact. The use of as simple a standard as a piece of colored glass certainly is a great advantage. The difference in color may not be so apparent to all eyes. The table accompanying the glass standard is an asset, as it definitely settles the question of time interval for development of the maximum color of acid hematin, and thus does away with one of the disadvantages of the earlier acid hematin methods.

Since the completion of this work a communication of Cohen and Smith (3) has appeared which confirms much of this work. They suggest the same standard solution because of its stability under army camp conditions.

CONCLUSIONS.

1. The Sahli hemoglobin method when using the color tubes accompanying the instrument gives very inaccurate results because of the decided variance in color density of the standard tubes, due to fading.

2. The Palmer method offers very satisfactory means of hemoglobin determinations if the standard solutions are freshly prepared. The method itself is very simple and may be successfully carried out by anyone familiar with colorimetry. The standard solutions prepared in the laboratory although carefully made have not been sufficiently stable to insure accurate determinations over periods of more than 3 to 4 weeks.

3. Newcomer's method obviates many difficulties heretofore observed with other procedures and gives good results with the glass 0.96 mm. in thickness, although the color is quite pale. When using the heavier glass, 1.02 mm. in thickness, the color match is only approximate and the figures obtained are not so satisfactory as those resulting from use of the thinner piece.

4. A method is presented applying Palmer's procedure to Sahli's principle which has proved most satisfactory. It removes the difficulty we encountered with Palmer's method, the lack of stability of color in the standard solutions. It has the advantage of an easier color match than that of red tint. The standards prepared have remained sufficiently unchanged for a period of 11 months to insure accurate hemoglobin determinations during this long period.

It may be suggested that for routine hospital work an acid hematin standard prepared in this way and kept at relatively constant temperature will remain unchanged for 8 months or longer. 1 per cent solutions may be prepared from time to time from the standard concentrated solution and this 1 per cent solution can be used to fill the standard tube of the common Sahli hemoglobinometer. This insures an accurate base line for hemoglobin determinations and with refilling of the Sahli tubes once a month will give accurate clinical determinations. Such clinical determinations are not the rule and are much to be desired.

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DIGESTIBILITY OF CERTAIN MISCELLANEOUS VEGETABLE FATS.*

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INTRODUCTION.

The shortage of food fats which existed during the war will, in all probability, be more or less permanent. For this reason it seemed desirable to obtain information on the nutritive value of some oils which have either not been used as foods or only used as such to a limited extent, but which might perhaps be thus used in case of need.

This office has made similar studies of the digestibility of about 50 more or less common fats and oils (1). The results seem to indicate that the digestibility coefficient of the fats having melting points above body temperature (37°) varies inversely with the melting point (2). It has also been found that most of the common oils are from 93 to 98 per cent utilized by the human body. Nevertheless, some oils were found to cause digestive disturbances and some oils are less tolerated by the body than others. Therefore, before we can say whether or not an oil or fat is suitable for use in the human dietary, we must know not only its coefficient of digestibility but also its effect on the human body when eaten in quantities at least equal to those in which butter or other common fat is used in the average dietary.

This paper records the digestibility of avocado and cupuassú bean fats, and cohune, hempseed, palm-kernel, and poppy-seed oils. Avocado fat is eaten largely in parts of the tropics, and

* Prepared under the direction of Dr. C. F. Langworthy, Chief, Office of Home Economics. Published with permission of the Secretary of Agriculture.

to some extent in this country as it occurs in the fruit; but the other oils studied have been used for food purposes only in a limited way, if at all, in this country. The study of the nutritive value of these oils seemed valuable not only on account of its interest in the field of nutrition but also on account of its very practical bearing upon our food supply, especially in times of shortage of the more common fats.

EXPERIMENTAL.

General Procedure.

The methods followed in the tests here reported are essentially the same as those followed in the previous digestion experiments conducted by this office. The fats, with the exception of avocado fat, were incorporated in a special corn-starch blanc-mange or pudding, and this was eaten with a basal ration consisting of a commercial wheat biscuit, oranges, and sugar, which supplied only a very small amount of fat in the total ration in comparison to that eaten in the blanc-mange. The avocado fat was eaten as it occurs in the fresh fruit pulp with a basal ration of milk and crackers, which supplied considerably less than one-half as much fat as was furnished by the avocados. Tea or coffee without cream was used if desired.

The amount of food eaten was accurately weighed and the weight of the water-dried feces for the experimental period was recorded. The methods for the separation of feces, analyses, etc. were the same as those reported in earlier papers (1).

The subjects who assisted in these tests were men, apparently in normal health, ranging in age from 20 to 40 years, who were students in local universities. They were familiar with this type of work and thoroughly trustworthy.

I. Avocado Fat.—The avocado (*Persea gratissima*) is a fruit indigenous to tropical and subtropical regions in the western hemisphere. In the United States it is cultivated to an increasing extent in Florida and California. The avocado, because of the large amount of fat it contains and because of its pleasant taste, seemed of interest to study. The avocados which we used in our experiments contained about 15 per cent fat, although Condit and Jaffa (3) state the average fat content to be about 20 per

cent. It is supposed to be present in the form of an emulsion, which probably accounts for the difficulty in expressing it from the pulp (Table I). Mattil (4), in two tests made during the progress of our experiments, using a basal ration of Graham crackers, cottage cheese, and milk, reports the digestibility of the total fat in a diet in which avacado fat was the predominating fat constituent as 93.7 per cent and 89.1 per cent. If the same correction which we made in our experiments for the fat of the diet of accessory foods is applied, the average of Mattil's figures becomes practically identical with the 88 per cent which we report for the digestibility of avocado fat alone. There were no

TABLE I.

Summary of Digestion Experiments with Avocado Fat in a Simple Mixed Diet.

Experiment No.	Subject.	Digestibility of entire ration.				Estimated digestibility of avocado fat alone.
		Protein.	Fat.	Carbohydrate.	Ash.	
		per cent	*per cent*	*per cent*	*per cent*	*per cent*
527	H. R. G.	85.0	90.8	96.1	74.9	88.3
529	P. K.	88.0	90.4	97.4	76.0	88.5
530	C. J. W.	84.5	88.7	97.6	73.7	86.8
Average		85.8	90.0	97.0	74.9	87.9

physiological disturbances caused by the ingestion of the avocado fat. The maximum intake by one subject was 124 gm. of avocado fat per day, while the average intake per man per day was 100 gm.

II. Cohune Oil.—Cohune oil is obtained from the kernel of the cohune palm (*Attalea cohune*), which grows quite extensively in Central America. The oil resembles coconut oil both in taste and odor, but has not been used very largely, due to the great difficulty experienced in breaking the very hard outer shell of the nut. At room temperature, the oil[1] has only a trace of solid fat. The subjects ate on an average of 52 gm. of cohune oil per man per day, while one subject ate 64 gm. per day for the experimental period (Table II). The oil was very well assimilated, being on an average 99 per cent digested, and produced no abnormal physiological effects.

[1] The sample used in this experiment was obtained through the courtesy of H. S. Bailey of the Bureau of Chemistry.

III. Cupuassú Fat.—The cupuassú fat is obtained by pressing the seeds which occur in the pulp of the fruit of the cupuassú tree (*Theobroma grandiflora* Schum), which is one of the most important fruit trees of Pará Brazil. Cupuassú fat has not been used for food purposes in this country. Nevertheless it seemed

TABLE II.

Summary of Digestion Experiments with Cohune Oil in a Simple Mixed Diet.

Experiment No.	Subject.	Digestibility of entire ration.				Estimated digestibility of cohune oil alone.
		Protein.	Fat.	Carbohydrate.	Ash.	
		per cent	*per cent*	*per cent*	*per cent*	*per cent*
621	A. A. F.	64.0	94.7	96.2	66.2	99.5
622	P. K.	68.8	94.9	97.0	80.0	98.6
623	J. C. M.	53.8	94.0	94.5	69.5	99.3
624	C. J. W.	67.3	94.6	95.6	77.5	98.8
Average		63.5	94.6	95.8	73.3	99.1

TABLE III.

Summary of Digestion Experiments with Cupuassú Fat in a Simple Mixed Diet.

Experiment No.	Subject.	Digestibility of entire ration.				Estimated digestibility of cupuassú fat alone.
		Protein.	Fat.	Carbohydrate.	Ash.	
		per cent	*per cent*	*per cent*	*per cent*	*per cent*
637	A. A. F.	78.4	91.0	97.4	63.3	95.3
638	P. K.	69.1	88.4	96.5	48.8	94.1
639	J. C. M.	77.5	89.6	96.1	54.2	95.0
640	C. J. W.	75.7	86.3	96.2	57.0	92.1
Average		75.2	88.8	96.6	55.8	94.1

to be a fat worth attention, since on account of the abundance of the cupuassú trees in Brazil it would be available in fairly large quantities.

It is a light yellow fat, solid at ordinary temperatures, and possessing the odor of cacao butter (Table III). The average digestibility, 94.1 per cent, found for cupuassú fat agrees very closely with that of cocoa butter, 94.7 per cent found in previous investigations (5). Only 41 gm. of cupuassú fat were consumed

on an average per man per day. The cupuassú fat caused slight physiological disturbances, such as nausea and looseness of the bowels, similar to those noted in the experiments with cocoa butter.

IV. Hempseed Oil.—Hempseed oil is expressed from the seeds of the hemp plant (*Cannabis sativa*). It is a yellowish green oil which has had practically no use as a food in this country, but has been almost wholly used for industrial purposes. It was eaten without aversion and caused no physiological disturbances. In our experiments hempseed oil was very completely assimilated, being on an average 98.5 per cent digested (Table IV). The subjects ate on an average 54 gm. per man per day, while one subject ate 57 gm. per day without any ill effects.

TABLE IV.

Summary of Digestion Experiments with Hempseed Oil in a Simple Mixed Diet.

Experiment No.	Subject.	Digestibility of entire ration.				Estimated digestibility of hempseed oil alone.
		Protein.	Fat.	Carbohydrate.	Ash.	
		per cent	*per cent*	*per cent*	*per cent*	*per cent*
906	J. F. C.	79.1	94.4	97.4	75.8	98.1
909	G. S. M.	56.4	94.7	96.3	65.0	99.5
910	W. O'C.	65.8	94.0	97.2	63.9	97.9
Average		67.1	94.4	97.0	68.2	98.5

V. Palm-Kernel Oil.—Palm-kernel oil is one of the two oils obtained from the fruit of an African palm tree (*Elaesis guineensis*). It is obtained by pressing the interior portion of the kernels. Palm oil, obtained from the exterior of the fruit, is rarely used for food purposes outside of the countries in which it is produced, but finds commercial application in the United States in the tin plate industry. The palm-kernel oil used in this experiment was obtained by cracking the kernels by hand and expressing the oil from the meats by a small sized, continuous process oil expeller. The oil possessed a pleasant odor and flavor much resembling coconut oil, but had a somewhat higher melting point, being a white solid at ordinary temperatures. The subjects ate on an average 100 gm. of palm-kernel oil per man per day

(Table V). The maximum intake was 121 gm. per day for the experimental period. The palm-kernel oil was very well assimilated, being 98 per cent utilized by the body, and it caused no physiological disturbances.

TABLE V.

Summary of Digestion Experiments with Palm-Kernel Oil in a Simple Mixed Diet.

Experiment No.	Subject.	Digestibility of entire ration.				Estimated digestibility of palm-kernel oil alone.
		Protein.	Fat.	Carbohydrate.	Ash.	
		per cent	*per cent*	*per cent*	*per cent*	*per cent*
685	A. A. F.	56.3	94.2	96.1	32.6	97.7
686	P. K.	81.0	93.9	99.2	82.7	95.1
687	J. C. M.	67.2	97.5	96.5	64.8	99.9
688	A. A. R.	39.3	95.7	95.6	45.7	99.3
Average		61.0	95.3	96.9	56.5	98.0

TABLE VI.

Summary of Digestion Experiments with Poppy-Seed Oil in a Simple Mixed Diet.

Experiment No.	Subject.	Digestibility of entire ration.				Estimated digestibility of poppy-seed oil alone.
		Protein.	Fat.	Carbohydrate.	Ash.	
		per cent	*per cent*	*per cent*	*per cent*	*per cent*
956	J. F. C.	62.4	92.8	96.1	57.3	96.1
957	F. K.	47.3	91.4	96.6	58.7	96.9
958	G. S. M.	67.0	93.4	96.7	56.7	98.5
959	W. O'C.	38.7	89.6	97.2	41.3	94.8
981	E. L. M.	58.0	89.2	96.4	47.7	95.1
982	G. S. M.	58.7	95.5	96.0	57.2	100.0
983	W. O'C.	11.5	87.4	96.5	32.7	92.9
Average		49.1	91.3	96.5	50.2	96.3

VI. Poppy-Seed Oil.—Poppy-seed oil is obtained by pressing the oil from the seeds of *Papaver album* and *Papaver nigrum*, two varieties of poppy, which are largely grown in the Orient. The best qualities of oil are used largely for edible purposes in Europe, but to a much smaller extent here. The best grades are used also for

artists' paints and other special technical purposes, while poorer qualities are used for soap-making. The oil used in these experiments was a good grade commercial oil which had a light yellow color and was without pronounced odor or flavor. It had the

TABLE VII.

Average Amount of Blanc-Mange and Total Food per Man per Day.

Diet.	Weight.	Water.	Protein.	Fat.	Carbohydrate.	Ash.	Fuel value.
	gm.	gm.	gm.	gm.	gm.	gm.	cal.
Avocado fat:							
Fat-rich avocado pulp...	688.4	547.8	6.0	99.8	30.0	4.7	1,042
Total food.....	1,603.4	1,194.9	43.0	153.9	198.2	13.5	2,350
Cohune oil:							
Fat-rich blanc-mange....	650.2	342.9	14.2	52.3	234.4	6.4	1,465
Total food.....	1,131.8	628.9	28.2	54.6	410.4	9.7	2,246
Cupuassú fat:							
Fat-rich blanc-mange....	536.0	310.9	22.5	39.9	159.5	3.2	1,087
Total food.....	902.6	496.5	36.3	42.0	321.6	6.1	1,810
Hempseed oil:							
Fat-rich blanc-mange...	406.8	177.2	7.3	53.5	165.7	3.1	1,174
Total food.....	1,026.2	538.5	21.2	55.8	403.9	6.7	2,205
Palm-kernel oil:							
Fat-rich blanc-mange....	577.7	251.9	9.8	100.3	213.0	2.7	1,785
Total food.....	1,001.2	490.7	22.9	102.4	379.5	5.6	2,531
Poppy-seed oil:							
Fat-rich blanc-mange.....	356.8	154.9	6.4	49.0	144.5	2.3	1,045
Total food.....	916.3	521.6	17.8	50.9	320.4	5.6	1,810

following constants: Iodine number 135.50, refractive index at 40°C. 63, and 1.24 per cent of free fatty acid. The subjects ate on an average 50 gm. of poppy-seed oil and one subject ate 60 gm. daily for the experimental period. The oil caused no physiological disturbances and was well assimilated by the body, being 96.3 per cent digested (Table VI).

Table VII reports the average amount of the fat-rich blancmange eaten per man per day and also the average total food consumed. Table VIII reports the coefficients of digestibility obtained by averaging the results of each group of experiments.

TABLE VIII.

Average Digestibility of Diet and Estimated Digestibility of Fats Studied.

Kind of fat.	No. of experiments.	Digestibility of entire ration.				Estimated digestibility of fats studied.
		Protein.	Fat.	Carbohydrates.	Ash.	
		per cent	*per cent*	*per cent*	*per cent*	*per cent*
Avocado fat	3	85.8	90.0	97.0	74.9	87.9
Cohune oil	4	63.5	94.6	95.8	73.3	99.1
Cupuassú fat	4	75.2	88.8	96.6	55.8	94.1
Hempseed oil	3	67.1	94.4	97.0	68.2	98.5
Palm-kernel oil	4	61.0	95.3	96.9	56.5	98.0
Poppy-seed oil	7	49.1	91.3	96.5	50.2	96.3

SUMMARY.

1. The digestibility of several fats and oils has been studied. The digestibility coefficients obtained were: Avocado fat, 87.9 per cent; cohune oil, 99.1 per cent; capuassú fat, 94.1 per cent; hempseed oil, 98.5 per cent; palm-kernel oil, 98.0 per cent; and poppy-seed oil, 96.3 per cent.

2. The digestibility of the protein and carbohydrate of the entire ration was essentially the same as that in other experiments of a similar nature, indicating that the fats exercised no unusual effect on the utilization of these constituents.

3. These fats and oils, with the possible exception of cupuassú fat, which caused slight disturbances, produced no abnormal physiological effects and may be regarded as satisfactory for food purposes. Cohune, hempseed, palm-kernel, and poppy-seed oils especially are very highly utilized by the human body.

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A METHOD FOR THE DETERMINATION OF METHEMOGLOBIN IN BLOOD.

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In the course of experimental work on the production of methemoglobin in pneumococcic infections it became desirable to have some simple method for the determination of methemoglobin in blood. The author was unable to find in the literature any method other than the complex and time-consuming spectrophotometric method, which requires an elaborate apparatus and is not very suitable when many determinations have to be made. The method outlined below is simple, quickly performed, and has given satisfactory results in a study of methemoglobin formation which will be published shortly.

Principle of the Method.—The method depends upon the fact that both hemoglobin and methemoglobin are changed quantitatively to cyanhemoglobin by dilute solutions of potassium cyanide. The color of the latter is a brilliant orange-red and is very suitable for colorimetric comparison. The change of methemoglobin to cyanhemoglobin is rapid, even in the cold. Hemoglobin, however, changes slowly at room temperature, and must be heated to 50°C. for about ½ hour for complete conversion. That the cyanhemoglobin from hemoglobin is identical with that from methemoglobin is shown by the identical absorption spectra in the two cases.

In practice the difficulty arising from the slow conversion of hemoglobin into cyanhemoglobin is avoided by converting all the hemoglobin present into methemoglobin by the use of a little potassium ferricyanide, and then converting the methemoglobin into cyanhemoglobin. The resulting solution of cyanhemoglobin is compared with a standard of known strength in a Duboscq colorimeter.

The total amount of hemoglobin plus methemoglobin having been thus determined colorimetrically, the hemoglobin content of the blood containing the two pigments (hemoglobin and methemoglobin) is determined separately from the oxygen capacity, employing the technique outlined in Van Slyke's[1] gasometric determination of hemoglobin. The hemoglobin determined by the oxygen capacity is subtracted from the hemoglobin plus methemoglobin determined together as cyanhemoglobin; the difference is the methemoglobin.

Details of the Method.—Oxalated whole blood is used. 2.00 cc. of the blood are placed in a 100 cc. flask and 50 cc. of water are added which effect hemolysis in a few seconds. 0.5 cc. of a 0.1 M (3.0 per cent) solution of potassium ferricyanide is added, and the

TABLE I.

Factors for Calculating Results from Analysis of 2 Cc. of Blood Saturated with Air.

Temperature.	Air physically dissolved by 2 cc. of blood. Subtract from gas volume to obtain corrected gas volume representing O_2 set free from hemoglobin.	Factor by which corrected gas volume is multiplied in order to give hemoglobin in 100 cc. of blood.
°C.	cc.	gm.
15	0.037	$34.7 \times \frac{B}{760}$
16	0.036	34.6 "
17	0.036	34.3 "
18	0.035	34.2 "
19	0.035	34.0 "
20	0.034	33.9 "
21	0.033	33.7 "
22	0.033	33.5 "
23	0.032	33.4 "
24	0.032	33.1 "
25	0.031	33.0 "
26	0.030	32.9 "
27	0.030	32.6 "
28	0.029	32.5 "
29	0.029	32.3 "
30	0.028	32.1 "

[1] Van Slyke, D. D., *J. Biol. Chem.*, 1918, xxxiii, 127.

flask allowed to stand for 15 to 20 minutes. (It was found that these conditions are optimum for the complete conversion of the hemoglobin to methemoglobin, only the faintest visible hemoglobin band being present at the end of 20 minutes with this amount of potassium ferricyanide.) 5 cc. of a 0.1 per cent potassium cyanide solution are now added. The change to cyanhemoglobin is immediate. Water is added to the mark and the solution compared with a standard of known strength in a colorimeter. The result is the hemoglobin plus methemoglobin, which we express as gm. of "total hemoglobin" per 100 cc. of blood.

A small portion (4 to 5 cc.) of the blood or hemoglobin solution is aerated in a funnel and its total oxygen capacity determined by the Van Slyke method. Barcroft[2] has shown that under these conditions (180 mm. of oxygen tension, 0 mm. of carbon dioxide tension, and room temperature) the hemoglobin is practically 100 per cent saturated. Therefore the oxygen capacity corresponds to the amount of hemoglobin present, and by dividing by 1.34 (the volume of oxygen combined with 1 gm. of hemoglobin) we obtain the gm. of hemoglobin per 100 cc. of blood. For convenience of calculation the factors for the conversion of cc. of gas combined with 2 cc. of blood into gm. of hemoglobin per 100 cc. of blood are given in Table I (modified from Van Slyke[1]).

Preparation of Standard.—The standard is prepared from fresh whole oxalated or defibrinated blood which is known to contain no methemoglobin. The hemoglobin content (gm. per 100 cc.) is determined gasometrically. 500 cc. of standard are made by placing 10 cc. of the blood in a 500 cc. flask, hemolyzing with about 300 cc. of water, and adding 2.5 cc. of the potassium ferricyanide solution. After 20 minutes, 25 cc. of the potassium cyanide solution are added and the mixture is diluted to the mark. The blood pigment value of this solution is known from the gasometric determinations and the unknown may be compared directly with it or suitable dilutions of the standard may be made.

[2] Barcroft, J., The respiratory function of the blood, Cambridge, 1914.

Calculation of Results.—An example will make this clear.

Strength of standard 15.0 gm. of hemoglobin per 100 cc. of blood.
Comparison of cyanhemoglobin in colorimeter; Standard 10, Unknown 12.
Unknown has $\frac{10}{12}$ of 15.0 or 12.5 gm. of total blood pigment per 100 cc.
Gasometric determination of hemoglobin 10.0 gm. per 100 cc.
Therefore, sample has 12.5−10.0 or 2.5 gm. of methemoglobin per 100 cc.

DISCUSSION.

The deep orange-red color of the cyanhemoglobin is adapted to accurate color comparisons. Fiftyfold dilution of normal blood, containing approximately 15 gm. of hemoglobin per 100 cc., gives about the optimum depth of color. A 1:100 dilution gives too light a color and requires that the standard be set at 20.

TABLE II.

Hemoglobin + methemoglobin observed per 100 cc. (Colorimetric.)	Hemoglobin calculated per 100 cc. (Gasometric).	Methemoglobin observed per 100 cc.	Methemoglobin calculated per 100 cc.
gm.	gm.	gm.	gm.
6.89	6.74	0.15	0.00
6.89	6.43	0.31	0.46
6.86	6.15	0.69	0.71
6.62	4.56	2.17	2.06
6.58	0.20	6.54	6.38

The author has attempted to determine methemoglobin colorimetrically in blood by converting it, with the hemoglobin, into carbon monoxide hemoglobin, for estimation by the Palmer[3] method, and into acid hematin for estimation by the Sahli principle; but both attempts were unsuccessful, since methemoglobin does not form products with carbon monoxide or acid which can be colorimetrically compared with the products formed from hemoglobin.

Results.—A solution of hemoglobin obtained from sheep's cells previously washed with saline was used. A gasometric determination of this solution showed it to contain 6.74 gm. of hemoglobin per 100 cc. Part of this solution was shaken for ½ hour with an amount of potassium ferricyanide calculated to change all of it

[3] Palmer, W. W., *J. Biol. Chem.*, 1918, xxxiii, 119.

to methemoglobin. The hemoglobin content of this solution (gasometric) was 0.20 gm. per 100 cc. These two solutions were mixed in various proportions so as to make solutions containing varying but known amounts of hemoglobin and methemoglobin. The methemoglobin content was then determined as outlined above, using a standard from ox blood which had been prepared a few days before. The results are shown in Table II.

CONCLUSIONS.

A colorimetric method for the determination of blood pigments is given which is simple and rapid, and which, combined with a simultaneous determination of the hemoglobin by the gasometric method of Van Slyke, gives the methemoglobin content of the blood.

A STUDY OF THE NEPHELOMETRIC VALUES OF CHOLESTEROL AND THE HIGHER FATTY ACIDS. II.

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Among the many influencing factors which govern the nephelometric value (N. V.) of fatty acids and cholesterol, I wish to discuss, in the present paper, certain important ones in their relation to saponification, because practically all quantitative fat determinations necessitate such a procedure. These factors consist of, on the one hand, substances which are essential to the determination, such as the reagents, or those which facilitate the production of colloidal suspensions, designated as protective colloids; and, on the other hand, conditions like heat, concentration, and the sequence of mixing the reagents in the development of turbidity.

However, before we apply either a procedure like saponification, or employ a protective colloid, we must ascertain its influence on the nephelometric values of the substances in question; an increase in the value is always to be desired, but a decrease is liable to render the determination less delicate, and therefore unsatisfactory as a micro method. Studies of this type are of interest not only as a preliminary step to the development of a new method, but also as a means of discovering the defects or proving the accuracy of a method already in use.

In the first paper,[1] where the suggested term nephelometric value was defined, the importance of acid concentration upon the nephelometric values of the fatty ingredients was emphasized and it was shown that fatty acids, as well as cholesterol, have specific nephelometric values. To overcome the difficulties caused by the fact that there exists a difference between the

[1] Csonka, F. A., *J. Biol. Chem.*, 1918, xxxiv, 577.

nephelometric values of the constituents of fat extracts, the first thought was to use a mixture for the standard, composed similarly to that of the unknown. The reliability of the result depends upon the extent of our success in reproducing the unknown mixture in the standard. Another suggestion would be to separate the ingredients of the fat extracts, although that would complicate the otherwise simple and rapid method. The ideal modification would be to employ such a technique as would render equal the nephelometric values of the various ingredients; the possibilities of regulating the nephelometric values by the factors referred to above will be demonstrated in the experimental part of this paper.

The present work is merely a preliminary study of the possibilities and as yet does not suggest any one procedure as preferable. The decision as to which of the above suggestions should be applied as the more useful in the nephelometric determination of fat will be considered in a later publication.

Methods.

Oleic acid and cholesterol were selected for the subjects of the present investigations, because they show the largest differences in their nephelometric values. The technique of these experiments, which is similar to that described in a previous article,[1] is such that the conclusions are directly applicable to the technique of Bloor's blood fat determination.

By the method described in the first paper,[1] the turbidity was produced by adding the HCl after the water had been added to the alcoholic solution of the fat; this was Technique A. In Technique B the sequence was reversed, the alcoholic solution of the fat being added to the water, and then the turbidity produced by adding the HCl as previously. In Series 1, oleic acid, cholesterol, and olive oil were used unmodified. In Series 2, the above mentioned substances were put through the saponification procedure by adding 2 cc. of N NaOH to the alcoholic solution in a 150 cc. beaker and placing this in a boiling water bath for 15 minutes to insure evaporation to dryness. To the residue were added 4 cc. of alcohol and the whole was warmed carefully to dissolve the fatty material. In those experiments, where the influence of gelatin upon the nephelometric value of oleic acid was studied, the amount of distilled water was substituted by a solution containing 50 mg. of gelatin. The final volume of the solution in which turbidity is produced is 50 cc. with 0.2 N acidity (HCl) and containing 4 cc. of 95 per cent alcohol. For comparison a permanent standard was employed, as previously described. Experiment A 1 with oleic acid was repeated many times during this work, as it is very im-

TABLE I.

Solution tested.	Amount.	Technique.	Turbidity produced.											
			5 min.		10 min.		15 min.		20 min.		25 min.		30 min.	
			Reading.	N. V.	Reading.	N. V.	Reading.	N. V.	Reading.	N. V.	Reading.	N. V.	Reading.	N. V.
	mg.		*mm.*		*mm.*		*mm.*		*mm.*		*mm.*		*mm.*	
Oleic acid.	2	A1	51.9	1.00	58.6	0.88	62.4	0.83	66.3	0.78	69.6	0.74	73.9	0.70
	2	A2	56.4	0.92	62.0	0.84	67.3	0.77	70.2	0.74	73.4	0.71	75.5	0.69
	2	B2*	50.2	1.03	56.5	0.92	60.4	0.86	63.0	0.82	64.0	0.81	66.5	0.78
Oleic acid and 50 mg. of gelatin.	2	A2	60.2	0.86	58.4	0.89	58.4	0.89	58.4	0.89	58.6	0.89	59.6	0.87
	4	B1	58.6	0.44	55.0	0.47	53.1	0.49	50.4	0.51	49.6	0.52	48.3	0.54
Cholesterol.	1	A1	49.6	2.09	48.0	2.16	47.9	2.17	47.3	2.19	47.0	2.21	47.6	2.18
	1	A2	51.1	2.03	49.4	2.10	48.2	2.15	48.1	2.16	48.7	2.13	49.2	2.11
	1	B1	48.3	2.15	47.1	2.20	46.4	2.23	46.3	2.23	46.4	2.23	46.4	2.23
	1	B2	44.3	2.34	44.3	2.34	44.1	2.35	44.1	2.35	44.1	2.35	44.9	2.31
Cholesterol and 50 mg. of gelatin.	2	B1	72.6	0.71	65.7	0.79	61.4	0.84	58.6	0.88	57.8	0.89	56.3	0.92

* B1 omitted as it is identical with A1.

portant to be sure that the intensity of the nephelometric field (permanent standard) remains unchanged throughout. Readings were taken every 5 minutes for ½ hour. The intensity of the nephelometric field used (permanent standard) is equal to the intensity of the turbidity produced by 2 mg. of the oleic acid after a lapse of 5 minutes, with the vernier set at the height of 51.9 mm. (Technique A, Series 1). The nephelometric values were calculated on that basis in all the experiments reported in this paper.

DISCUSSION.

As a result of the investigation described in the previous paper,[1] we conclude that in quantitative determinations it is erroneous to compare materials having different nephelometric values. From the experiments shown in Table I, it is evident that the saponification procedure alters the nephelometric value of the substance, and therefore it is advisable to treat the standard in the same manner as the unknown. In his first publication, Bloor[2] advocated the use of unsaponified triolein as a standard, later substituting for this oleic acid. By so doing, he overlooked the first source of error and disregarded the second.

The nephelometric value depends upon the conditions under which turbidity is produced; on account of the inconsistent and contradictory behavior of the different substances, the technique which is the more desirable must be previously determined in each individual case. For instance, we find that the nephelometric values of oleic acid and cholesterol in Series 1 are practically the same, whichever technique is employed, while Technique B gives higher values for Series 2. On the other hand, if we compare the result on olive oil[3] which was selected to represent the glycerides in general (Table II), we find very little difference in Series 2, while Series 1 shows a pronounced difference; the values being much higher according to Technique A than to B.

By observing the changes of the nephelometric values within certain time intervals, it is interesting to note the difference between the changes shown by oleic acid and cholesterol; namely, the nephelometric value of the former is highest at the first reading and then decreases, while that of the latter shows a con-

[2] Bloor, W. R., *J. Biol. Chem.*, 1914, xvii, 377; 1915, xxiii, 317.

[3] The olive oil was dissolved in an alcohol-ether mixture for the stock solution.

TABLE II.

Solution tested.	Amount.	Technique.	Turbidity produced.											
			5 min.		10 min.		15 min.		20 min.		25 min.		30 min.	
			Reading.	N. V.	Reading.	N. V.	Reading.	N. V.	Reading.	N. V.	Reading.	N. V.	Reading.	N. V.
	mg.		*mm.*		*mm.*		*mm.*		*mm.*		*mm.*		*mm.*	
Olive oil.....	2	A1	51.3	1.01	56.9	0.91	60.2	0.86	62.3	0.83	65.9	0.79	68.2	0.76
" "	2	A2	57.5	0.90	63.9	0.81	69.0	0.75	72.3	0.72	76.6	0.68	78.7	0.66
" "	2	B1	61.8	0.84	68.5	0.76	75.0	0.69	80.4	0.65	85.6	0.61	88.6	0.60
" "	2	B2	57.8	0.90	65.3	0.79	70.4	0.74	73.2	0.71	76.6	0.68	81.4	0.64

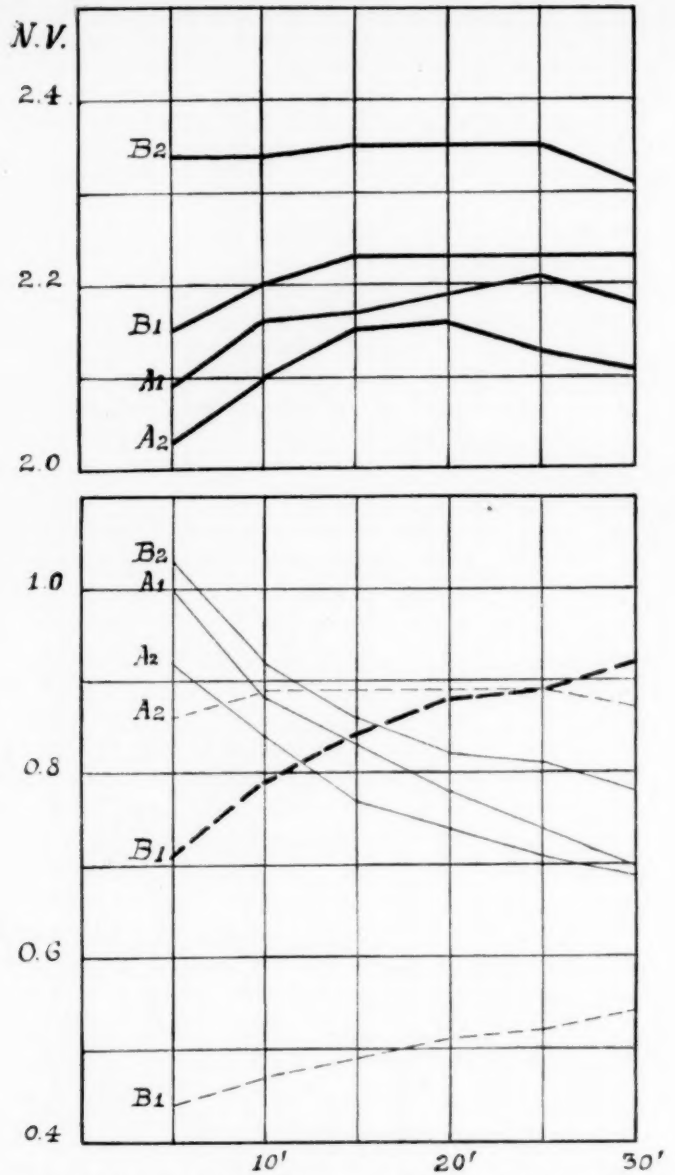

CHART 1. Changes of the nephelometric values (N. V.) within the first half hour.

Cholesterol ——— Oleic acid ———
" with gelatin — — — " " with gelatin — — —

tinuous increase throughout the 30 minute interval. Chart I presents this variation graphically.

That it is possible to change not only the height, but also the entire direction of the curve is exemplified by the use of gelatin as a protective colloid, as first suggested by Murlin. With oleic acid (Series 1) the nephelometric value is more than 50 per cent lower after a lapse of 5 minutes and while this steadily increases it does not attain its maximum height within the 30 minute interval. In Series 2 the nephelometric values range between narrow limits, and are but slightly lowered. Determinations A1 and B2 were not successful; fairly constant readings could be obtained only in case of A2 and B1. Furthermore, applying Technique A the author was unable to produce a fine suspension of cholesterol in the presence of gelatin, even when the cholesterol was mixed with oleic acid, as the former separated into clumps on the surface. The presence of gelatin decreases the nephelometric value of cholesterol over 60 per cent when the turbidity is produced according to Technique B. A bluish tint is always observed in the nephelometric field whenever gelatin is used, which makes the reading somewhat difficult.

SUMMARY.

Oleic acid and cholesterol were the subjects of investigation. The nephelometric values of these substances were found to be influenced by the saponification procedure as well as by the addition of certain substances which alone, under similar circumstances, do not produce any turbidity. These influencing agents are exemplified by the use of gelatin as a protective colloid.

THE HEPTOSES FROM GULOSE AND SOME OF THEIR DERIVATIVES.

By F. B. La Forge.

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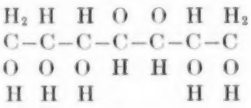
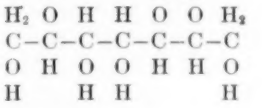
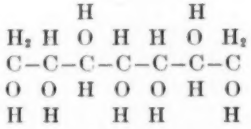
(Received for publication, December 31, 1919.)

Of the sixteen theoretically possible heptoses of the *d* series, the following, as well as some of their derivatives, have been prepared and studied; α-glucoheptose,[1] β-glucoheptose,[1] α-mannoheptose,[2] β-mannoheptose,[3] α-galaheptose,[3] and β-galaheptose.[3] The number of possible heptitoles, if optical isomerism is disregarded, obtained by reduction of these sugars is only ten, since the same heptitol may be formed from two different sugars. Thus, Peirce[4] showed the identity of α-*d*-mannoheptitol with α-*l*-galaheptitol.





Likewise β-galaheptitol should be identical with one of the heptitols from gulose.



In the present paper the preparation from gulose of two new heptoses and their derivatives will be described and the identity of one of these with β-*d*-galaheptitol established.

[1] Fischer, E., *Ann. Chem.*, 1892, cclxx, 64.

[2] Fischer, E., and Passmore, F., *Ber. chem. Ges.*, 1890, xxiii, 2226.

[3] Fischer, E., *Ann. Chem.*, 1895, cclxxxviii, 139.

[4] Peirce, G., *J. Biol. Chem.*, 1915, xxiii, 327.

EXPERIMENTAL.

α- and β-d-Guloheptonic Acids from Gulose.

80 gm. of gulose from the carefully purified hydrazone[5] were dissolved in 200 cc. of water and slightly more than the calculated amount of hydrocyanic acid was added together with a few drops of ammonia to catalyze the reaction. The solution soon began to warm up, showing that reaction was taking place. After about 8 hours 25 gm. of sulfuric acid, diluted with a small amount of water, were added and the reaction products allowed to stand over night.

The solution was then strongly diluted, 150 gm. of barium hydroxide were added to it, and the ammonia was expelled by boiling. The barium was precipitated by a very slight excess of sulfuric acid and the barium sulfate removed by filtration with suction. The resulting solution contained the mixture of α- and β-guloheptonic acids. These could be obtained as a colorless syrup by decolorizing and evaporating the solution.

Barium Salt of α-Guloheptonic Acid.

For the preparation of this derivative the solution obtained from the above mentioned amount of gulose in 200 cc. of water was exactly neutralized while warm with a concentrated solution of barium hydroxide and then allowed to stand in the ice box. After several days the crystallization of the barium salt was judged to be complete. The yield was about 100 gm. The mother liquor contained the barium salt of the β-acid which will be referred to later. It was recrystallized from about twenty parts of hot water, from which it separates in large colorless plates generally grouped in rosettes.

[5] The crude gulose obtained by reduction of gulonic lactone with sodium amalgam (Fischer, E., and Piloty, O., *Ber. chem. Ges.*, 1891, xxiv, 521) was dissolved in about eight parts of 65 to 70 per cent alcohol and to this solution was added slightly more than the calculated amount of phenylhydrazine base. Crystallization of the hydrazone began at once and was complete after several hours. The voluminous crystalline mass, which is difficult to filter, was subjected to strong pressure between several layers of filter paper and the resulting press-cake recrystallized from alcohol.

0.3550 gm. of substance gave 0.1402 gm. of $BaSO_4$.

	Calculated for $C_{14}H_{28}O_{14}Ba$.	Found.
	per cent	*per cent*
Ba	23.37	23.22

Its optical activity could not be measured owing to its low solubility but seemed to be very slight.

α-d-Guloheptonic Acid.

The barium salt was dissolved in a convenient amount of hot water, the barium removed quantitatively with sulfuric acid, and the barium sulfate removed by filtration with suction. Upon concentration of the resulting solution the acid was obtained as a colorless syrup which did not crystallize. For reduction to the sugar the syrup was heated for a short time on the steam bath in order to convert as much as possible into the lactone.

Phenylhydrazide of α-Guloheptonic Acid.

This derivative was obtained very easily by heating a concentrated solution of α-guloheptonic acid, with about the theoretical amount of phenylhydrazine, on the steam bath for 1 hour. Upon cooling the reaction product crystallizes out and may be purified by recrystallization from 75 per cent alcohol. It forms long white needles which melt at 191–192°, uncorrected.

2.0656 gm. of substance in 25 cc. of H_2O rotated in a 2 dm. tube with D-light $-5.0°$ $[\alpha]_D^{20} = -15.38°$.

α-d-Guloheptose.

30 gm. of the mixture of acid and lactone were dissolved in eight to ten parts of cold water and reduced with sodium amalgam.[6] The reduction required about 200 gm. of 2.5 per cent amalgam. The solution was maintained acid by addition of dilute sulfuric acid from time to time.

The sodium salts were removed by pouring the hot concentrated solution into hot 95 per cent alcohol. The alcoholic solution was concentrated to a syrup, which was again taken up in a large volume of 95 per cent alcohol in order to separate the sugar as far as possible from the sodium salts.

[6] La Forge, F. B., and Hudson, C. S., *J. Biol. Chem.*, 1917, xxx, 68.

The alcoholic solution was then concentrated *in vacuo* to a small volume.

Crystallization of the sugar took place during evaporation. The yield was very good (9.0 gm.). It may be recrystallized from two parts of hot water or from ten parts of 60 per cent alcohol and forms rosettes of long needles. It is thus much less soluble than most sugars and is only slightly sweet. The sugar melts at 185–187°, uncorrected.

0.7616 gm. of substance in 25 cc. of H_2O rotated in a 2 dm. tube at 20° and D-light after about 8 minutes − 19.5° to the left, after 35 minutes − 14.0°, and became constant at − 11.5°. $[\alpha]_D^{20} = -65.65°$.

0.1703 gm. of substance gave 0.1017 gm. of H_2O and 0.2521 gm. of CO_2.

	Calculated for $C_7H_{14}O_7$. per cent	Found. per cent
C	40.00	40.36
H	6.66	6.57

α-Guloheptitol.

5 gm. of α-guloheptose were reduced with sodium amalgam in the usual manner. The solution was kept slightly alkaline most of the time. When the copper reducing power had practically disappeared, which required 3 days action of the reagent, the reaction product was poured into hot alcohol to remove the sodium sulfate and the solution concentrated to a syrup. This crystallized, on rubbing with absolute alcohol, to a semisolid, waxy mass. After washing with alcohol the product was dissolved in three or four parts of water and hot absolute alcohol added in amount sufficient to produce permanent turbidity.

Under these conditions the compound crystallized in rosettes of hard prisms, which melted at 138–141°, uncorrected. After a second recrystallization it showed no change in melting point.[7]

[7] Peirce[4] has prepared β-galaheptitol and gives the melting point at 141-144° after the substance had begun to soften at 138°. He states that the melt did not become clear until the temperature was raised to 190°. From the method used by him for the preparation of β-galaheptitol, it seems extremely likely that his product may have been contaminated with some of the epimeric α-galaheptitol. There seems to be no reason to suppose that β-galaheptitol and α-guloheptitol are not identical.

0.1009 gm. of substance gave 0.1480 gm. of CO_2 and 0.0682 gm. of H_2O.

	Calculated for $C_7H_{14}O_7$. *per cent*	Found. *per cent*
C	39.60	39.96
H	7.64	7.51

1.3280 gm. of substance in 25 cc. of H_2O showed no rotation in a 2 dm. tube which could be detected.

1.0624 gm. in 25 cc. of saturated aqueous borax solution rotated in a 4 dm. tube with D-light + 0.68° to the right.

β-Guloheptonic Acid and β-Guloheptose.

The filtrate from α-guloheptonic acid barium salt, above referred to, was further concentrated and cooled to cause the separation of as much of the α-compound as possible. The filtrate was diluted, the barium removed quantitatively with sulfuric acid, and the solution of the β-acid, together with some of the α-compound, concentrated to a syrup. This syrup was heated on the steam bath to convert as much as possible of the acid into lactone and then reduced under the conditions already described.

After separation of the sodium salts by means of twice repeated treatment with hot 98 per cent alcohol the sugar was obtained upon evaporation of the solvent as a syrup. This syrup still contained some of the α-compound which could be removed to a large extent by dissolving the mixture in a small amount of glacial acetic acid and allowing the almost insoluble α-sugar to crystallize out.

The syrup resulting after removal of the acetic acid was not entirely pure but was used in this condition for reduction.

Reduction of β-Guloheptose.

The syrupy β-guloheptose was reduced according to the usual method in slightly alkaline solution. The reduction was continued until the copper reducing power of the solution had almost completely disappeared, after which the salts were removed and the reaction product obtained as a syrup. Since this syrup showed no tendency to crystallize, it was transformed into the benzal derivative.

Benzal-β-Guloheptitol.

This derivative was obtained with ease by agitating equal parts of 70 per cent sulfuric acid, benzaldehyde, and the syrupy mixture containing the heptitol. It was washed with water and alcohol and dried at 100°. It melted at about 260° with decomposition.

β-Guloheptitol.

One part benzal-β-guloheptitol was boiled for about 1½ hours with about twelve parts 60 per cent acetic acid. The solution was diluted and excess of the reagent, together with most of the benzaldehyde, was removed by repeated extractions with ether. It was then concentrated to a small volume and again extracted with the same solvent. Upon standing the syrup resulting from evaporation of the final solution deposited crystals of the heptitol which were washed with glacial acetic acid and recrystallized from 95 per cent alcohol. The compound melted at 129°, uncorrected. It was again recrystallized and melted at 128–129°, uncorrected.

0.1025 gm. of substance gave 0.1495 gm. of CO_2 and 0.0653 gm. of H_2O.

	Calculated for $C_7H_{16}O_7$. *per cent*	Found. *per cent*
C	39.60	39.98
H	7.64	7.09

0.3175 gm. of substance in 5 cc. of saturated aqueous borax solution showed no appreciable rotation in a 1 dm. tube.

STUDIES ON THE SECRETION OF GASTRIC JUICE.

By SEYMOUR J. COHEN.

(From the Laboratory of Pharmacology and Therapeutics, College of Medicine, University of Illinois, Chicago.)

(Received for publication, December 2, 1919.)

In a previous article (1), on the secretion of gastric juice in fever, it was reported that the concentration of chlorides in gastric juice was constant or slightly reduced. In this present paper I wish to emphasize the relation between the secretion of total chlorides in gastric juice and the secretion of free hydrochloric acid. Most of the data for this paper were obtained at that time but until now have remained unpublished.

The gastric juice was obtained, as previously, from the stomach of dogs with a Pavlov pouch. The animals were kept on a standard diet. Their temperature varied as the result of the production of fever by intravenous injection of solutions of sodium nucleinate or *Bacillus prodigiosis*. The free hydrochloric acid was determined by titrating 1 cc. of the gastric juice with N/40 NaOH, dimethylaminoazobenzene being used as indicator. The total chlorides were determined by the method of McLean and Van Slyke (2).

The results given were obtained from 175 chloride determinations of gastric juice on seventeen different animals. The free hydrochloric acidity varied from 0.0 to 0.48 per cent while the total chlorides varied from 0.39 to 0.54 per cent. In examining Charts 1, 2, 3, and 4, it will be noted that the curves for the secretion of free hydrochloric acid do not correspond to the curves for the secretion of total chlorides, although the percentage of total chlorides is always greater than the percentage of free acid, as should be expected. The curve for the total chlorides is almost a straight line with just slight deviations, while the curve for the free acid varies markedly. In some places, however, both curves run nearly parallel, while at other times there is practically no relation between them.

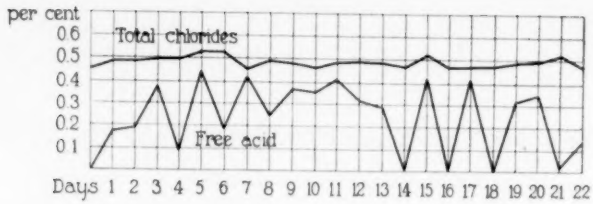


CHART 1. Showing the relation between the secretion of free HCl and the secretion of total chlorides on Dog 2.

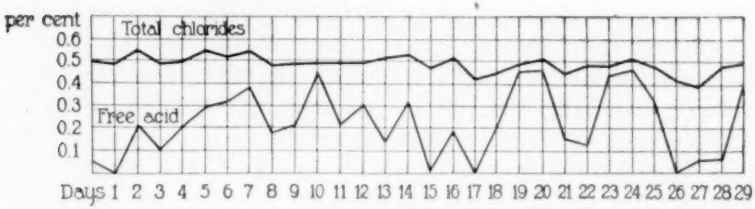


CHART 2. Showing the relation between the secretion of free HCl and the secretion of total chlorides on Dog 5.

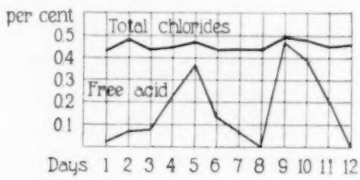


CHART 3. Showing the relation between the secretion of free HCl and the secretion of total chlorides on Dog 20.

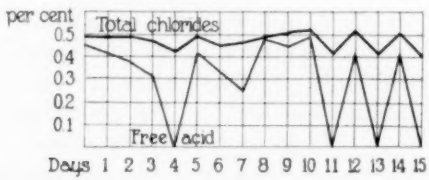


CHART 4. Showing the relation between the secretion of free HCl and the secretion of total chlorides on Dog 22.

From these results it appears that the chlorides of gastric juice are secreted at a more or less constant rate and in all likelihood independently of the hydrochloric acid secretion. In other words you may get just as high a percentage of total chlorides in a sample of gastric juice when the free acidity is 0.0 as when it is 0.35 per cent. This may indicate that the hydrochloric acid is not secreted as such but is probably formed by the secretion of some substance, containing the chlorine ion, which is transformed in the lumen of the stomach or by stomach mucosa to the free acid. This, however, is not a new idea (3). If free hydrochloric acid were secreted as such by the gastric glands, one would expect that with a decrease in the free acidity or a total absence of the free acidity there would be associated with it a marked fall in the total chlorides, but this does not seem to be true. The chlorides are secreted more or less constantly regardless of the free acidity of the gastric juice.

On the other hand, these results may coincide with those of Pavlov (4), and Rehfuss and Hawk (5). Pavlov states that the gastric juice as it flows from the glands possesses a constant acidity; variations are due to secondary neutralization. The apparent constant rate of secretion of chlorides may mean that the gastric juice is secreted with a constant acidity and that the variations in the end-product are due to neutralization by mucus or the alkalinity of the stomach mucosa. This point might be proved by studying a case of achylia and determining the chloride content in the juice; such a case has not been available.

Another factor in the secretion of HCl is the relation between volume and acidity. Pavlov states that, when secretion of the gastric gland is abundant, then the acidity is higher than when the secretion is scant. This is due to the fact that when the juice flows rapidly the acidity is higher, because there is less change of the mucus or alkaline mucosa to neutralize it. This fact is very well illustrated by Charts 5, 6, and 7 in which the volume of gastric juice (5 hours secretion) is charted with the free acidity. It will be noted that free acidity is almost directly proportional to the total volume. This agrees with Pavlov's statement that a large volume of gastric juice, which is secreted rather rapidly, has a higher acidity than a smaller volume, which is secreted more slowly.

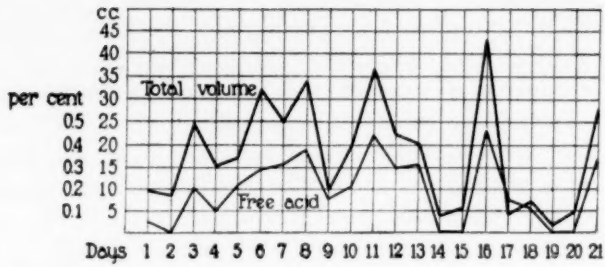


CHART 5. Showing the relation between the secretion of free HCl and the total volume on Dog 2.

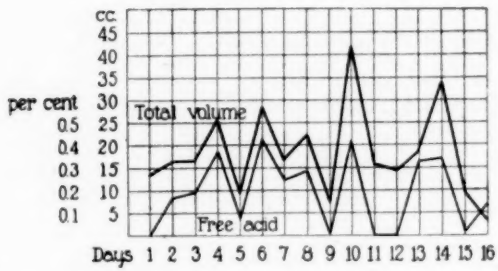


Chart 6. Showing the relation between the secretion of free HCl and the total volume on Dog 5.

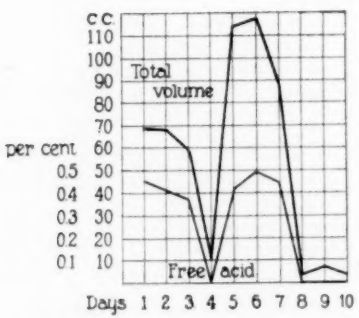


CHART 7. Showing the relation between the secretion of free HCl and the total volume on Dog 22.

SUMMARY.

1. The total chlorides of gastric juice from the dog are secreted more or less constantly regardless of the free acidity of the gastric juice. They vary from 0.39 to 0.54 per cent.

2. This may corroborate Pavlov's view that gastric juice is secreted with a constant acidity and that variations are due to secondary neutralization.

3. A large volume of juice secreted rapidly has a higher acidity than a smaller volume secreted slowly, other factors remaining constant (confirmatory of Pavlov).

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DIRECT QUANTITATIVE DETERMINATION OF POTASSIUM AND SODIUM IN SMALL QUANTITIES OF BLOOD.

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(Received for publication, January 2, 1920.)

INTRODUCTION.

When a solution of a cobalt salt in dilute acetic acid is added to a solution of sodium nitrite the following reaction is said to occur:

$$21\ Co(C_2H_3O_2)_2.\ 4\ H_2O + 14\ NaNO_2 + 4\ H(C_2H_3O_2) = Na_6\ [CO_2(NO_2)_{12}]\ H_2O + 8\ NaC_2H_3O_2 + 2\ NO + 6\ H_2O.$$

The solution becomes dark brown and oxides of nitrogen are evolved. When these are removed by aeration and a little of the resulting reagent is added to a fairly concentrated solution of any potassium salt containing a little acetic acid, an orange-yellow precipitate is formed at once. The following reaction is presumed to take place (1):

$$Na_6CO_2\ (NO_2)_{12}.\ H_2O + 2\ K_2SO_4 = 2\ K_2Na\ [CO\ (NO_2)_6]\ H_2O + 2\ Na_2SO_4.$$

These phenomena were first reported by de Koninck (2) and a few months later by Curtman (3). They observed independently that the reagent does not react with Ca, Mg, Na, Ba, Sr, Zn, Fe, sulfate, nitrate, or chloride ions. It is therefore possible to detect potassium in the presence of these ions. The ammonium ion gives a precipitate with the reagent and phosphates, unless present in very small amounts, will do likewise. Addie and Wood (4) isolated and analyzed the potassium sodium cobalti-nitrite compound formed under definite experimental conditions. They showed that the precipitate has a constant composition represented by the formula $K_2Na\ [Co\ (NO_2)_6]\ H_2O$, and determined its solubility to be rather a little less than 1 part in 20,000. If the concentration of the potassium salt is not less than 0.5 per cent, the precipitate is formed at once, settles rapidly, and the particles are large enough to be retained by a Gooch filter.

Where it is desired to determine large amounts of potassium as the cobalti-nitrite compound, the method of Addie and Wood (4), that of Drushel (5), or some modification of either may be used (1, 6, 7). The extreme insolubility of the precipitate in water, its sensitiveness under appropriate conditions for minute amounts of potassium, and the indifference of the reagent to other ions usually present in physiological fluids make it suitable for the determination of such small amounts of potassium as are found in blood.

The first attempt to determine potassium in small amounts of blood as the potassium sodium cobalti-nitrite compound was made by Hamburger (8). By his method precipitation is accomplished in a centrifuge tube, the lower end of which is drawn out to a capillary and graduated. The volume of the precipitate is measured in this tube, each graduation being equal to 0.0001 gm. of potassium. The precipitation requires at least 16 hours for completion. No analyses of serum, plasma, or blood are given.

Greenwald (7) has described a composite method for the determination of potassium, sodium, calcium, and phosphates in 90 gm. of blood. The potassium is precipitated as the cobalti-nitrite compound. More recently Clausen (9) reported a method for the determination of potassium in small quantities of blood. The figures reported justify all that the author claims for his method. The precipitation method is essentially that advocated by Drushel. In some preliminary experiments which we performed in 1917 this procedure was tried. In agreement with Bowser (10) it was found that in attempting to concentrate the fluid after adding the reagent decomposition of the precipitate at times took place, resulting in the formation of what appeared to be cobalt acetate. Hence we discarded it for the simpler procedure described below.

The methods described in this paper were devised for the study of changes in the concentration of sodium and potassium in the blood of children. The results of these studies, carried on for more than 2 years, will be published later. In the meanwhile it has been thought desirable to put the methods themselves on record.

Potassium Method.

1 cc. of blood, 3 to 5 cc. of clear plasma, or an equal amount of serum is dried in a platinum dish over the steam bath, then in the incubator at 110°C. for about ½ hour. The dish or crucible is then placed in a flat-bottomed quartz dish, 10 cm. in diameter and 6 cm. deep, in the bottom of which are placed several pieces of porcelain. The outer dish is then heated with the low flame of a large Meker burner until fumes begin to come off. The heating is continued until no more fumes are given off, when the flame is turned on full until the charred material is immobile. The large dish is then covered with a quartz plate and heating continued until the material is completely ashed.[1] The platinum dish is

[1] The ashing often proceeds rapidly at first, then some residual carbon is left which does not readily become oxidized. At this stage the ash is dissolved in a little concentrated hydrochloric acid, evaporated over the steam bath, then dried in the incubator at 105°C., and heated until a white crystalline ash is obtained.

then removed, allowed to cool, and the ash dissolved in 0.5 cc. of water with the aid of one or two drops of glacial acetic acid. 0.5 to 1 cc. of sodium cobalti-nitrite reagent is then added, drop by drop, with stirring and the mixture allowed to stand for at least 10 minutes. During this time a Gooch crucible is prepared. One or two pieces of hardened filter paper are placed at the bottom, then asbestos emulsion is poured in, and the pad sucked dry. When finished the pad should be at least 2 mm. in thickness and should be washed with a large quantity of water. The precipitate is transferred to the wet pad. The suction should be regulated so that the water runs through drop by drop; the rest of the precipitate is washed onto the pad with small portions of cold water. It is then washed repeatedly with small portions of water until the washings return perfectly clear. Precipitate and pad are then transferred *en masse* to a 50 cc. beaker, and the crucible is washed with a little water.[2] The paper is then removed with a forceps and washed clean with distilled water. Not more than 10 cc. of water need be used for complete transference of asbestos pad and precipitate. 25 cc. (an excess) of 0.01 N potassium permanganate and 5 cc. of 25 per cent sulfuric acid are added. The mixture is stirred, heated over the steam bath for just 3 minutes, and sufficient 0.01 N oxalic acid is then added to decolorize the solution completely. The material is at once titrated back to a permanent pink with 0.01 N $KMnO_4$ solution. The total number of cc. of 0.01 N $KMnO_4$ − number of cc. of 0.01 N oxalic acid × 0.071 = mg. of potassium in the sample.

A blank may be done but the correction is insignificant as a rule.

Details of the Procedure.

Collecting Sample.—Blood is collected by puncture of the median basilic, median cephalic, or external jugular vein. Whole blood is collected directly into a weighed platinum crucible and rapidly weighed. Plasma or serum may be collected under oil as in the Van Slyke and Cullen determination of the CO_2-combining power of blood plasma (11). To obtain plasma, potassium-free am-

[2] The pad need not be sucked dry. By means of a glass rod it may be dislodged by a half turn and can then be transferred as a whole including the entire precipitate.

monium oxalate or oxalic acid may be used as anticoagulant. Although ammonium salts react with sodium cobalti-nitrite, the ammonia is completely volatilized during the ashing. To obtain serum, the blood is collected under oil in a centrifuge tube and allowed to remain in the ice box until the serum has separated. If the blood is collected in this manner or in a clean, dry test-tube the danger of hemolysis will be reduced to a minimum.[3]

Ashing.—The method of ashing which has already been described was first used by Stolte (12). It is simple and fairly rapid. There is no loss of salts through volatilization. Chemically pure potassium or sodium chloride may be thus heated for 48 hours without loss. 1 mg. of potassium similarly treated may be recovered quantitatively. It is important to do such control tests to be sure that the experimental conditions have been duplicated.

Asbestos.—Dilute permanganate solutions when heated on the water bath for some time undergo change. This process is hastened by the presence of asbestos and is not due to organic matter that may be present in the asbestos. (This is all oxidized in a preliminary blank titration.) Nevertheless, if the oxidation is not continued beyond 3 minutes as described, the presence of the asbestos has no influence on the titration. We have found the use of barium sulfate suspension superfluous and its presence makes transference of the precipitate more difficult. Asbestos emulsion may be made by prolonged digestion of a good asbestos with strong nitric and hydrochloric acid for about 8 hours. The asbestos is separated from the supernatant fluid by filtering through a Buchner funnel. It is then washed with water until washings are no longer acid to litmus, then suspended in water, digested with 10 per cent NaOH for an equal period of time, filtered off, washed with dilute hydrochloric acid and then with water, sucked dry, suspended in water, and shaken until a uniform suspension is obtained.

[3] Owing to the large amount of potassium present in corpuscles even a moderate amount of hemolysis introduces considerable error.

Preparation of the Reagents.

25 per cent sulfuric acid is made by diluting chemically pure concentrated sulfuric acid with water. It should be tested with permanganate for the presence of organic matter.

The Sodium Cobalti-Nitrite Reagent.—This may be prepared according to the method of Addie and Wood (4). A somewhat easier method is the following, first suggested by Hamburger (8). This yields a less concentrated reagent.

Solution A.—50 gm. of cobalt nitrate crystals (J. T. Baker) are dissolved in 100 cc. of water and to this solution 25 cc. of glacial acetic acid are added.

Solution B.—50 gm. of C.P. sodium nitrite (potassium-free) (J. T. Baker) are dissolved in 100 cc. of water. Mix six volumes of Solution A and ten volumes of Solution B. An evolution of nitric oxide gas occurs at once. Air is drawn through the solution until all the gas has passed off. The reagent is then allowed to stand in the ice box for at least 24 hours. It is best filtered before using.

The cobalt nitrate and sodium nitrite need be weighed only roughly. If kept in the ice box the reagent will keep for at least a month and often much longer. Although 1.5 cc. of reagent will precipitate as much as 20 mg. of potassium (Hamburger), we have found it best to use about 0.5 cc. where 1 mg. of potassium or less is thought to be present. For quantities between 1 to 3 mg., 1 cc. of reagent will suffice.

0.01 N Potassium Permanganate Solution.—0.01 N potassium permanganate solution was prepared from a N or 0.1 N solution by appropriate dilution. The solution was standardized against a known 0.01 N oxalic acid solution. The latter was made from a N oxalic acid solution.

Na, Ca, Mg, Fe, sulfates, chlorides, and nitrate do not interfere. Ammonia must be removed. Although such small amounts of phosphates as occur in normal blood do not interfere with the accuracy of the potassium determination, nevertheless, as has recently been shown (13), the inorganic phosphates may be markedly increased in the blood serum in certain pathological conditions. Hence it may be necessary to remove them (see Table II). The following method has been found satisfactory. The solution

of blood or serum ash acidified with hydrochloric acid is heated on the steam bath for a few minutes. 2 cc. of 2 per cent $BaCl_2$ solution are added drop by drop followed after a few minutes by 0.5 cc. of concentrated ammonia. 15 cc. of a saturated solution of ammonium carbonate in a mixture of equal volumes of concentrated ammonia and 95 per cent alcohol (14) are added drop by drop with stirring. After ½ hour the precipitate, which contains practically all the barium, calcium, magnesium, sulfate, and phosphate, is filtered through ash-free filter paper and washed several times with the precipitating reagent.

When the filtrate which contains the sodium and potassium has been evaporated to a small volume, a few drops of concentrated hydrochloric acid are added. Evaporation to dryness is continued and completed in the incubator at 110°C. The residue is then heated to constant weight by the method of Stolte and the sodium and potassium are weighed as the chlorides. When combined with the cobalti-nitrite method for potassium, this procedure may be used as a rapid method for the indirect determination of sodium.[4]

Protocols.

Three solutions were made up.

Solution 1 contained 1 mg. of potassium per cc. as chemically pure potassium chloride.

Solution 2 had the following composition:

	gm.
NaCl	6.4389
KCl	1.4184
$Na_2HPO_4.2H_2O$	2.4992
$MgSO_4$	0.3695
$CaCl_2$	0.1836
H_2O up to 1,000.0 cc.	

Solution 3 contained

	gm.
NaCl	1.287
KCl	0.56
Ca as $CaCl_2$	0.10
Mg as $MgSO_4$	0.20
H_2O up to 200.0 cc.	

All chemicals were "Kahlbaum zur Analyse" or prepared by recrystallization of "Baker's Analyzed." Na_2HPO_4, $2H_2O$ was recrystallized and air-dried (17).

[4] It is also possible to weigh the combined chlorides of sodium and potassium, determine the total chlorine, and calculate the amount of potassium and sodium (15, 16).

TABLE I.

Analysis of Four Samples of Solution 1.

Sample No.	K present.	K found.
	mg.	*mg.*
1	1.00	0.99
2	1.00	0.98
3	1.00	1.00
4	1.00	1.01

TABLE II.

Analysis of Six Samples of Solution 2.

Sample No.	K present.	K found.*
	mg.	*mg.*
1	0.74	0.84
2	0.74	0.84
3	2.22	2.45
4	2.22	2.45
5	1.00	1.13
6	1.00	1.07

* The high results are no doubt due to the presence of phosphate. Solution 3 contains same ingredients but no phosphates.

TABLE III.

Analysis of Three Samples of Solution 3.

Sample No.	K present.	K found.
	mg.	*mg.*
1	1.00	0.98
2	1.00	1.05
3	1.00	1.06

TABLE IV.

Recovery of Potassium Added to Serum.

	K present.	K found.
	mg.	*mg.*
K in serum	0.94	
K added as KCl	1.48	
Total	2.42	2.39
K in serum	0.94	
K added as KCl	1.48	
Total	2.42	2.42

Sodium Method.

Introduction.

The usual method for determining sodium in the presence of other ions is to isolate sodium and potassium as chlorides or sulfates, weigh the combined salts, then determine potassium as the chloroplatinate, the cobalti-nitrite, or the perchlorate, and calculate the amount of sodium from the data thus obtained. Such methods are indirect, very tedious, and obviously cannot be used to determine small quantities.

The general solubility of sodium compounds in the common solvents has tended to discourage attempts to devise a direct sodium method. Fenton (18) found that the sodium salt of dihydroxytartaric acid is insoluble in cold water and used this as the basis for a direct sodium method. Although recommended by Sutton,[5] the method has not attained any degree of popularity probably because of the difficulty in preparing the reagent and the solubility of the precipitate.

In 1910 Ball (19) described a direct method for the determination of sodium based on the formation of an insoluble sodium cesium bismuthi-nitrite ($9\ CsNO_2{,}6NaNO_2{,}5Bi(NO_2)_3$). The reagent is expensive, does not keep well, and large quantities must be used for each determination. The precipitation must be accomplished in a special vessel in the absence of air and requires 48 hours for completion. No suitable washing solution has been found which does not dissolve some of the precipitate.

The use of potassium pyroantimonate for the detection of sodium has been known for a long time. When a solution of this reagent is added to a fairly concentrated solution of a sodium salt, a precipitate is formed, either at once or on standing, which is crystalline in nature and has the composition $Na_2H_2Sb_2O_7$, $6\ H_2O$ (20). Precipitation is favored by neutral or slightly alkaline reaction, and is accelerated by the addition of alcohol. The precipitate is very slightly soluble in water. The presence of K, Mg, Ca, SO_4, PO_4, or chloride ions does not interfere with the reaction. *Ammonium salts also form an insoluble pyroantimonate* and must therefore be removed.

[5] Sutton (15), p. 65.

Technique of the Sodium Method.

The ash of 1 or 2 cc. of blood, serum, or plasma obtained as described for the potassium method is dissolved in water, in a platinum dish, using 0.5 cc. of water for each cc. of serum, plasma, or blood. Solution may be aided by the addition of a drop or two of N hydrochloric acid. The solution is then made slightly alkaline with freshly prepared 10 per cent KOH solution. 15 cc. of the reagent and one-fifth of the entire volume of absolute alcohol[6] are then added. Precipitation occurs at once. The mixture is stirred, allowed to stand for at least 2 hours (preferably over night), then transferred to the wet pad of a weighed Gooch crucible as in the potassium method. It is then washed four or five times with 3 cc. portions of 30 per cent alcohol, dried at 110°C., cooled in a desiccator, and weighed. 1 mg. of sodium yields 11.08 mg. of precipitate. All reagents should be tested for the presence of sodium and ammonium salts, especially the potassium hydroxide. A blank determination should be done and the result subtracted from the Na determination in the sample. Most of the laboratory reagents will give a slight precipitate with the potassium pyroantimonate reagent.

Preparation of the Reagents.

Potassium pyroantimonate (J. T. Baker, C.P. analyzed chemicals), 2 gm. of the powder, is added to 100 cc. of boiling water in a 350 cc. Pyrex flask and heating continued until no more dissolves. It is then cooled rapidly under the tap and 3 cc. of 10 per cent KOH are added, and the solution is stirred and filtered. The clear filtrate constitutes the reagent.[5] Although I have been able to precipitate sodium with a reagent that had been kept in the ice box for 2 months, I have, nevertheless, always prepared the solution fresh each time. The potassium antimonate need be weighed only roughly and the rest of the preparation takes but a few minutes. 10 per cent KOH should preferably be free of both sodium and ammonium salts. Alcohol-washed KOH contains relatively little of these. The exact content, if any is

[6] The addition of too much alcohol will precipitate some of the reagent which is itself not very soluble and hence the results will be too high.

TABLE V.

Analysis of a Solution of C. P. Sodium Chloride.

Sodium as sodium chloride.	
Present.	Found.
mg.	*mg.*
5.91	5.87
5.91	6.02

TABLE VI.

*Analysis of Samples of Solution 2.**

Sodium.	
Present.	Found.
mg.	*mg.*
6.34	6.53
6.34	6.48
6.34	6.40
6.34	6.43

	gm.
*NaCl	6.4389
KCl	1.4184
$Na_2HPO_4,2H_2O$	2.4992
$MgSO_4$	0.3695
$CaCl_2$	0.1836

H_2O up to 1,000.0 cc.

TABLE VII.

Recovery of Sodium Added to Serum.

	Present.	Found.
	mg.	*mg.*
Sodium in serum	14.90	
" added as sodium chloride	6.34	
Total	21.24	22.32
Sodium in serum	14.90	
" added as sodium chloride	6.34	
Total	21.34	21.16
Sodium in serum	19.40	
" added	6.34	
Total	25.74	25.30

present, should, of course, be determined and a correction made for the amount used. This solution should also be made fresh or kept so that it will neither absorb ammonia nor dissolve sodium.

CONCLUSIONS.

Simple methods for the direct quantitative estimation of sodium and potassium in small quantities of blood, serum, or plasma have been described that are usually accurate to within 3 per cent of the theory and often closer (see Tables I, III, V, and VI).

By using the indirect sodium method described above, both sodium and potassium may be determined on 3 cc. of serum with an error not exceeding ± 5 per cent.

Results obtained by both methods for sodium are in satisfactory agreement.

The potassium content of normal human serum varies between 16 and 22 mg. per 100 cc. of serum. The sodium content has been found in both normal children and adults to vary between 280 and 310 mg. per 100 cc. of serum.

Sodium or potassium added to serum may be recovered almost quantitatively (see Tables IV and VII).

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